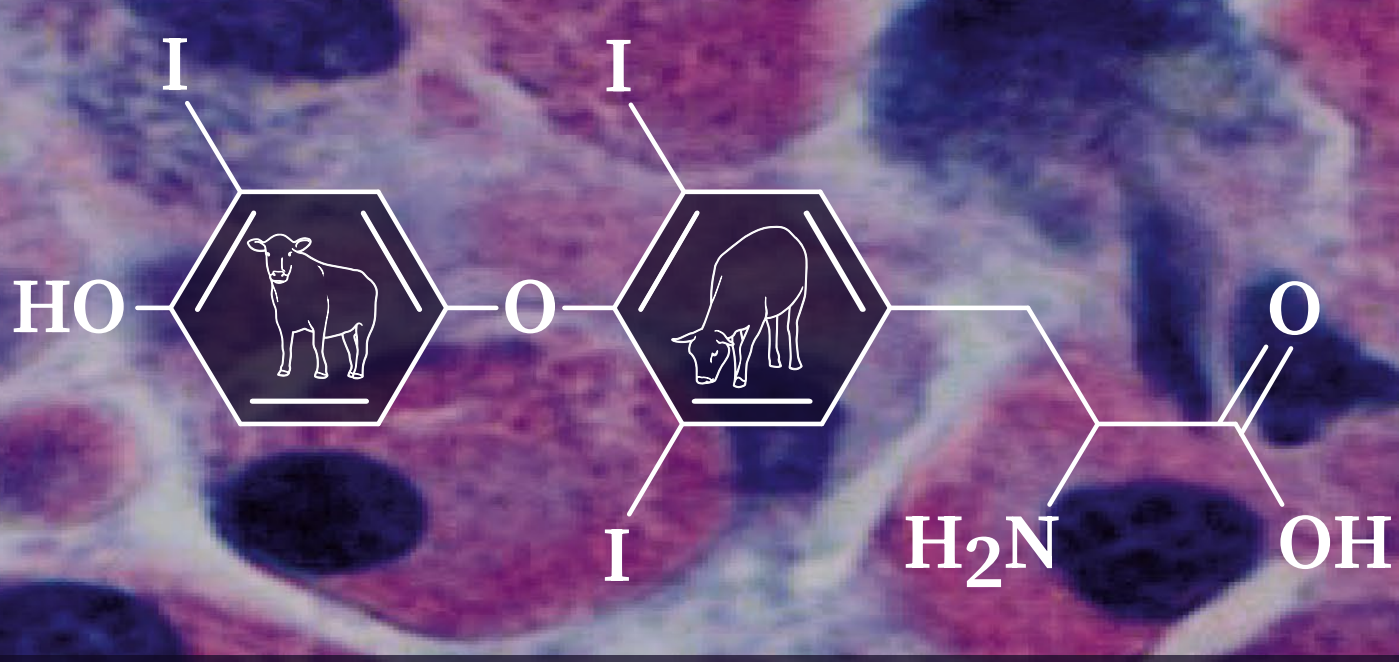
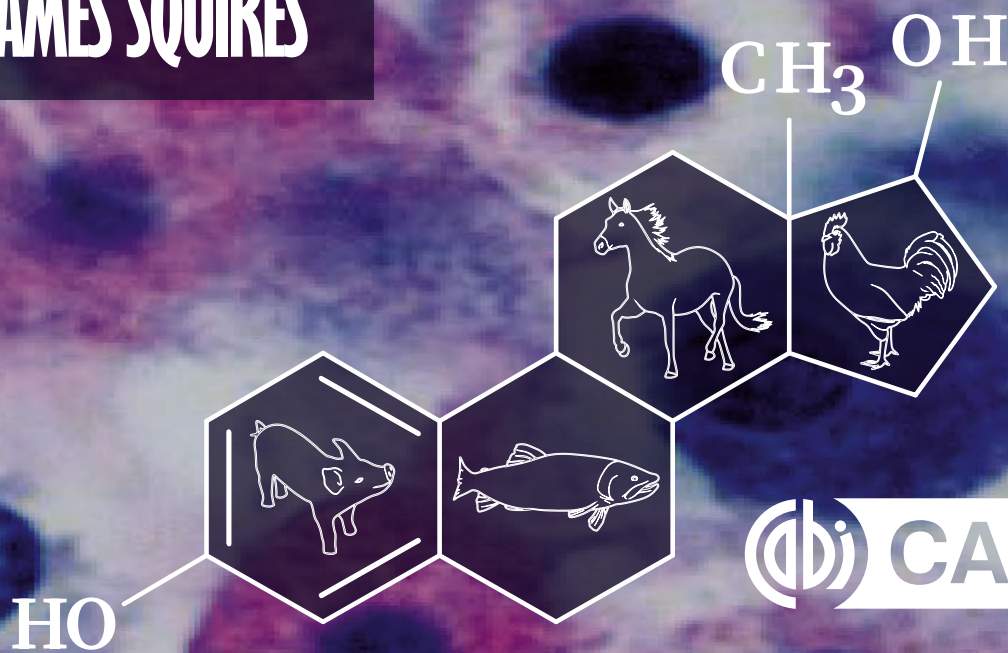


APPLIED ANIMAL ENDOCRINOLOGY

3rd Edition

E. JAMES SQUIRES



CABI

Applied Animal Endocrinology, 3rd Edition

To Gail, Allison, Victoria and Kimberly,
and in loving memory of Dorothy Cavell Squires,
Eli James Squires (Sr) and Gary Ernest Lodge.

Applied Animal Endocrinology, 3rd Edition

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Contents

Preface to the 3rd Edition

This new edition is a refresh of the 2nd edition to bring the information up to date and to highlight new areas of endocrinology that have potential applications in commercial animals. Figures and text have been updated throughout to include new material and current references, and some areas are covered in more detail with more examples included. Chapter 1 includes more details and updated information on G protein receptors, the function of CREB, methods for identification of DNA regulatory sequences and DNA-binding proteins, circadian rhythm and the biological clock. Chapter 2 has expanded coverage of *in vitro* models to include 3D cell culture and organ-on-a-chip, updated information on gene editing, antibody production, hormone delivery methods and DNA cloning and sequencing methods. Chapter 3 has an update on boar taint and a new section on the gut microbiome and the effects of antibiotics and antimicrobials. In Chapter 4 there is a new section on skin as an endocrine organ. Chapter 5 includes an update on current methods in reproduction and Chapter 6 has updated information on endocrine disruptor testing methods. I hope that this new edition will prove useful to those in academia and industry and, as always, constructive criticism will be most welcome.

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Preface to the 2nd Edition

The purpose and focus of this edition of *Applied Animal Endocrinology* remain the same as for the first edition: that is, to describe and integrate the principles of the endocrine systems that affect animal production, reproduction, health and welfare. The overall organization of the text is unchanged, but I have updated the information in all the chapters, corrected errors and addressed many of the suggestions made in the published reviews of the first edition. In particular, Chapter 3, which covers the endocrine systems that affect growth rate, feed efficiency and carcass composition, has been extensively reorganized, and the section on the regulation of food intake has been expanded. I have also included an expanded list of questions for study and discussion, improved the index and updated the reading list. I hope that this new edition will prove useful to interested individuals in academia and industry and stimulate new ideas in applications of endocrinology to animal production systems. As always, constructive criticism will be most welcome.

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Preface to the 1st Edition

The purpose of this book is to provide information on a number of different endocrine systems that affect animal production and to describe how these systems can be manipulated or monitored to advantage. A number of excellent endocrinology texts are already available that describe the function of various endocrine systems, but these texts, for the most part, deal with human or comparative endocrinology. This book focuses on commercial animals, and endocrine systems that can affect growth and carcass composition, the production of animal products (milk, eggs and wool), reproduction efficiency, and animal health, behaviour and welfare are described. Detailed information on the mechanism of action of the endocrine systems is covered, and an attempt is made to integrate knowledge from similar topics by focusing on common mechanisms and themes (for example, see the discussion of dietary polyunsaturated fatty acids (PUFA) in Chapter 3, Section 3.7). This information is used to understand potential methods for altering these systems and, hopefully, to stimulate ideas for the development of new methods.

The first two chapters cover the essential background information in endocrinology, my version of 'Endocrinology 101', but also include information on the production of hormones and the methods for manipulating endocrine systems. In the remaining chapters, endocrine systems that affect some aspect of animal production are described. Each chapter includes an overview of the problem or application, followed by a description of the endocrine systems affecting the problem and a discussion of how these systems can be manipulated or monitored to advantage.

In Chapter 1, the structure and function of hormones and receptors are covered. The main concepts of endocrinology are reviewed in sufficient depth to provide the necessary background material for the rest of the book. An attempt was made to avoid excessive detail and to introduce some potential applications to heighten interest. An initial overview of hormones and endocrinology is followed by a discussion of the synthesis, release and metabolism of hormones, the intracellular and extracellular mechanisms of hormone action and the integration of hormone action at the level of the hypothalamus and pituitary.

Chapter 2 covers various endocrine methodologies. The methods that are used to study how endocrine systems function are described, followed by methods for measuring hormones and receptors. Methods used for the production of hormones are then described and, finally, a number of methods for manipulating hormone function are covered.

In Chapter 3, endocrine systems that affect growth rate, feed efficiency and carcass composition are described. This includes anabolic steroids and analogues, use of uncastrated (intact) male pigs and the problem of boar taint, somatotrophin, β-adrenergic agonists, thyroid hormones, dietary PUFA (linoleic, linolenic, γ-linolenic acid (GLA) and conjugated linoleic acid (CLA)), leptin, control of appetite by cholecystokinin (CCK), antibiotics, antimicrobials and other factors, dietary chromium and insulin, and the effects of stress on meat quality.

In Chapter 4, the endocrine effects on animal products other than meat are covered. These include endocrine effects on mammary gland development and milk production (including the regulation of mammogenesis, lactogenesis and galactopoiesis), the effects of bovine somatotrophin (bST), the factors affecting milk composition, and metabolic diseases related to lactation. This is followed by a discussion of endocrine effects on egg production, including those on ovary sexual development in chickens, and the regulation of follicular development and eggshell formation. Finally, wool production and endocrine defleecing are covered.

Chapter 5 is concerned with the endocrine manipulation of reproduction. In the first section, sexual differentiation and maturation of mammals are covered, followed by the regulation of the oestrous cycle, pregnancy and parturition. Methods for manipulating reproduction are then discussed, including manipulation of the oestrous cycle, pregnancy, the postpartum interval, inducing puberty and advancing cycling in seasonal

breeders. The next section covers endocrine manipulations in aquaculture, including control of reproduction, effects on growth and nutrient utilization and the effects of stress.

In Chapter 6, the applications of endocrinology in monitoring and manipulating animal behaviour, health, performance and welfare are described. The control of broodiness in turkeys and applications of pheromones in vertebrates and insects are discussed first. This is followed by a section on the effects of stress, including the hormonal responses to stress and the effects of stress on immune function, reproduction and growth performance. The endocrine applications in toxicology are covered in the final section, which illustrates that changes in endocrine function can be used as indicators of endocrine disruptors in the environment and food chain.

This text is aimed at senior undergraduate and graduate students in animal science and veterinary medicine, although others in academia and industry who are interested in applications of endocrinology in animal production systems should also find it useful. It is based on a course – Applied Endocrinology – that has been taught at the Department of Animal and Poultry Science, University of Guelph, for the past 15 years. It is my hope that it will help to integrate knowledge of endocrine function in commercial animals and stimulate new ideas and applications for improving animal production, health and welfare. Constructive criticism and comments will be most appreciated.

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Acknowledgements for the 2nd Edition

Special thanks are due to Kimberly Jen for preparation of the figures, Rebecca Brill for preparation of the index and Gregoy Bédécarrats and John Walton for helpful suggestions on sections of the manuscript.

Acknowledgements for the 1st Edition

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Acronyms and Abbreviations

$1,25(OH)_2D_3$	1,25-hydroxyvitamin D_3 (1,25-dihydroxycholecalciferol)
3-NOP	3-nitrooxypropanol
3MOI	3-methyloxindole
3MI	3-methylindole (skatole)
6-OH-3MI	6-hydroxyskatole
9-HDA	9-hydroxydec-2-enoic acid
9-ODA	9-oxodec-2-enoic acid
11-KT	11-ketotestosterone
17α,20β-DP	17α,20β-dihydroxy-4-pregnen-3-one
17β-HSD	17β-hydroxysteroid dehydrogenase
β-AA	β-adrenergic agonists
β-GAL	β-galactosidase
β-LPH	β-lipotrophin
AA	arachidonic acid
Ab	antibody
ACC	acetyl CoA carboxylase
ACTH	andrenocorticotrophic hormone
ADG	average daily gain
ADH	antidiuretic hormone (vasopressin)
ADI	acceptable daily intake
AdipoR	adiponectin receptor
ADSF	adipose tissue-specific secretory factor, FIZZ3, resistin
ADRA2A	α2A-adrenergic receptor
AF-2	activation function-2 region
Ag	antigen
AGP	antimicrobial growth promoters
AgRP	agouti-related peptide
AhR	aryl hydrocarbon receptor
AI	artificial insemination
AIS	androgen insensitivity syndrome (testicular feminization, Tfm)
AKR1C	aldo-keto reductase 1C
Akt	AKR mouse strain thymoma (also known as protein kinase B (PKB)
ALA	α-linolenic acid (18:2, *n*-3)
ALP	alkaline phosphatase
ALS	acid labile subunit
AMH	anti-Müllerian hormone
AMHRII	anti-Müllerian hormone receptor
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	activating protein-1
APPL1	adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper-containing protein 1
AR	androgen receptor (NR3C4)

ARA	androgen receptor co-activator
ARC	arcuate nucleus (of the hypothalamus)
AREs	androgen response elements
ARIP	androgen receptor interacting protein
AT	adenine-thymine
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCIP/NBT	5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium
BHB	β-hydroxybutyrate
BLAST	basic local alignment search tool
BMP	bone morphogenic protein
BMPR	bone morphogenic protein receptor
BMR	basal metabolic rate
BPA	bisphenol A
BRAC	basic rest–activity cycle
bsAB	bispecific antibody
bST	bovine somatotrophin
BW	body weight
CAD	cation–anion difference
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CAP	6-chloro-8-dehydro-17-acetoxy-progesterone
CAP1	adenylyl cyclase-associated protein 1
CAR	constitutive androstane receptor
CART	cocaine- and amphetamine-related transcript
Cas	CRISPR-associated nuclease
CASI	cell autonomous sex identity
CaSR	calcium-sensing receptor
CAT	chloramphenicol acetyltransferase
CBG/CBP	corticosteroid-binding globulin (corticosteroid-binding protein, CBP, transcortin)
CCK	cholecystokinin
CCKAR	cholecystokinin A receptor
CCKR	cholecystokinin receptor
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
ChIP	chromatin immunoprecipitation
ChIPseq	chromatin immunoprecipitation and sequencing
CIDR	controlled internal drug-releasing device
CL	corpus luteum
CLA	conjugated linoleic acid
ClpB	caseinolytic protease B
CNDPL	cytosol non-specific dipeptidase
CNP	chlornitrofen, 2,4,6-trichlorophenyl-4′-nitrophenyl ether
CNS	central nervous system
CNTs	carbon nanotubes
ConA	concanavalin A
COX	cyclo-oxygenase
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CRC	calcium release channel (ryanodine receptor, Ryr)
CREB	cAMP-responsive element-binding protein
CRH (CRF)	corticotrophin-releasing hormone (factor)

CRHR	corticotrophin-releasing hormone receptor
CRISPR	clustered regularly interspaced short palindromic repeats
CrNic	chromium nicotinate
CRP	C-reactive protein
CrPic	chromium picolinate
crRNAs	CRISPR-RNAs
Cr-Tf	Cr-transferrin
CRY	cryptochrome protein
cST	chicken somatotrophin
CV	coefficient of variation
CYB5	cytochrome b5
CYP	cytochrome P450
CYP11A1	cytochrome P450 side-chain cleavage
CYP17A1	cytochrome P450C17A1
CYP19	cytochrome P450 aromatase
CYP24A1	cytochrome P450 24-hydroxylase
DAG	diacylglycerol
DAX1	dosage-sensitive sex-reversal–adrenal hypoplasia critical region of the X chromosome
DBD	DNA-binding domain
DDT	dichlorodiphenyltrichloroethane
DCAD	dietary cation–anion difference
DCN	decorin
DES	diethylstilboestrol
DFD	dark, firm and dry (meat)
DFM	direct-fed microbials, probiotics
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	5α-dihydrotestosterone
DIO	deiodinase
DMN	dorsomedial nucleus
DMRT1	DM domain-related transcription factor 1
DPP-IV	dipeptidyl peptidase IV
DSMZ	German Collection of Microorganisms and Cell Cultures
DT	diphtheria toxin
E_2	oestradiol
EAR	ErbA-related
EAR2	ErbA-related protein 2
EBI	ergosterol biosynthesis-inhibiting (fungicide)
ECACC	European Collection of Cell Cultures
ECD	extracellular domain
eCG	equine chorionic gonadotrophin (pregnant mare serum gonadotrophin, PMSG)
ECHA	European Chemical Agency
ECL	extracellular loop
ECM	extracellular matrix
ECP	oestradiol cypionate
EDCs	endocrine disruptor chemicals
EDSP	Endocrine Disruptor Screening Program
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority

EGF	epidermal growth factor (urogastrone)
EGFR	epidermal growth factor receptor (ErbB1)
EGTA	ethylene glycol tetra-acetic acid
ELISA (EIA)	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
eNOS	endothelial NOS
EPA	eicosapentaenoic acid
EPO	erythropoietin
ER	endoplasmic reticulum
ERα and ERβ	oestrogen receptor α and β
ErbB	erythroblastic oncogene B
ERE	oestrogen-responsive element
ERK	extracellular signal-regulated kinase (mitogen-activated protein kinase, MAPK)
EROD	7-ethoxyresorufin-*O*-deethylase
ERR	oestrogen-related receptor
ESP	exocrine gland-secreting peptide
EST	expressed sequence tag
FA	fatty acid
FAO	Food and Agriculture Organization of the United Nations (UN)
FAS	fatty acid synthase
FEBP	feto-neonatal oestrogen-binding protein
FFAR2	free fatty acid receptor 2
FGA	fluorogestone acetate
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FIA	fluorescence immunoassay
FID	flame ionization detector
FIL	feedback inhibitor of lactation
FIZZ3	found in inflammatory zone 3
FMT	faecal microbiota transplants
FNDC5	fibronectin type III domain-containing protein 5
FOS	fructo-oligosaccharides
FOXL2	forkhead transcription factor 2
FPR	formyl peptide receptor
FSH	follicle-stimulating hormone
FXR	farnesoid X receptor
G protein	GTP-binding protein
GC	gas chromatography
GCNF	germ cell nuclear factor
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GFP	green fluorescent protein
GH	growth hormone (somatotrophin, ST)
GHBP	growth hormone binding protein
GHIH	growth hormone release-inhibiting hormone (somatostatin, SS)
GHRH	growth hormone-releasing hormone
GHRP	growth hormone-releasing peptide
GHS	growth hormone secretagogue
GHS-R	growth hormone secretagogue receptor
GIT	gastrointestinal tract

GLA	γ-linolenic acid
GLP	glucagon-like peptide
GLUT	glucose transport protein
GLUT4	glucose transporter 4
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMOs	genetically modified organisms
GnIH	gonadotrophin-inhibitory hormone; gonadotrophin release-inhibiting hormone
GnIHR	gonadotrophin-inhibitory hormone receptor
GnRH	gonadotrophin-releasing hormone
GnRHR	gonadotrophin-releasing hormone receptor
GO	graphene oxide
GOS	galacto-oligosaccharides
GPCR	G protein-coupled receptor
GPER1	G protein-coupled membrane-bound oestrogen receptor
GR	glucocorticoid receptor
GRAS	generally recognized as safe
GRIP1	glucocorticoid receptor interacting protein 1
GRK	GPCR-regulating kinase
GRP	gastrin-releasing peptide
GRPP	glicentin-related pancreatic peptide
GTF	glucose tolerance factor
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HAT	hypoxanthine–aminopterin–thymidine (medium)
hCG	human chorionic gonadotrophin
HDAC	histone deacetylase
HDL	high-density lipoprotein
HDR	homology-directed repair
H-FABP	fatty acid-binding protein from heart (mammary-derived growth inhibitor, MDGI)
HGF	hepatocyte growth factor
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HIT	histidine triad
HMG	high-mobility group binding (proteins)
HMOI	3-hydroxy-3-methyloxindole
HMT	histone methyltransferase
HMW	high molecular weight
HNF	hepatic nuclear factor
HOB	methyl *p*-hydroxybenzoate
HPA	hypothalamic–pituitary–adrenal (axis)
HPI	hypothalamic–pituitary–interrenal (axis)
HPLC	high-performance liquid chromatography
HREs	hormone-responsive elements
HSD3B	3β-hydroxysteroid dehydrogenase
Hsp90	90 kDa heat shock protein
hST	human somatotrophin
HVA	4-hydroxy-3-methoxyphenylethanol
I3C	indole-3-carbinol
IASPs	inhibitors of the activin receptor signalling pathway
IBMX	isobutylmethylxanthine
ICD	intracellular domain
ICL	intracellular loops

ICS	immunochromatographic strip
IFN	interferon
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1 (somatomedin)
IGFBP	insulin-like growth factor-binding protein
IL	interleukin
IMO	isomalto-oligosaccharides
ImP	imidazole propionate
IPM	integrated pest management
iNOS	inducible NOS
IP_3	inositol 1,4,5-phosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
IPA	indole-3 propionic acid
IPM	integrated pest management
IR	insulin receptor
IRM	integrated resource management
IRS	insulin receptor substrate
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
JAK	Janus kinase
JECFA	Joint Expert Committee on Food Additives
KGF	keratinocyte growth factor
KISS1	kisspeptin
KISS1R	kisspeptin receptor (GPR54)
KLH	keyhole limpet haemocyanin
LA	linoleic acid (18:2, *n*-6)
Lac-Phe	*N*-lactoyl-phenylalanine
LaH	lateral hypothalamus
LBD	ligand-binding domain
LBP	ligand-binding pocket
LD50	lethal dose for 50% of test animals
LDL	low-density lipoprotein
L-DOPA	3,4-dihydroxyphenylalanine
LEP	leptin
Lep-R	leptin receptor, Ob-R
LH	luteinizing hormone
LIF	leukaemia inhibitory factor
LMWCr	low-molecular-weight chromium-binding substance (chromodulin)
LOD	limit of detection
LOQ	limit of quantification
LPH	lipotropin hormone
LPS	lipopolysaccharide
LR8	lipoprotein receptor with 8 ligand-binding domains
LRH-1	liver receptor homologue 1
LT	leukotriene
LTB	enterotoxin subunit B
LUC	luciferase
LUC-NE	nerve fibres of the locus ceruleus that secrete noradrenaline (norepinephrine)
LXR	liver X receptor
MAC-T3	mammary alveolar cell transfected with large T antigen
MALDI–TOF	matrix-assisted laser desorption ionization–time of flight (mass spectrometry)

MAP	6-methyl-17-acetoxy-progesterone
MAPK	mitogen-activated protein kinase (extracellular signal regulated kinase, ERK)
mAR	membrane-bound AR
MAS	meiosis-activating sterol
MCH	melanin-concentrating hormone
MCP-1	monocyte chemotactic protein-1
MCR	melanocortin (MSH) receptor
MCSF	macrophage colony-stimulating factor
MCT8	monocarboxylate transporter 8
MDGI	mammary-derived growth inhibitor (fatty acid-binding protein from heart (H-FABP))
MGA	melengesterol acetate
MH	malignant hypothermia
MHC	major histocompatibility complex
MIH	melanocyte-stimulating hormone release-inhibiting factor
miRNAs	microRNAs
MIS	maturation-inducing steroid
MOE	main olfactory epithelium
MOET	multiple ovulation and embryo transfer
MPF	maturation promoting factor
MPGF	major proglucagon-derived fragment
mPR	membrane progesterone receptor
MR	mineralocorticoid receptor
MRF	melanocyte-stimulating hormone-releasing factor
MRI	magnetic resonance imaging
MRL	maximum residue level
MS	mass spectrometry
MSH	melanocyte-stimulating hormone (melanocortin)
MTMT	male urine thiol, (methylthio)-methylthiol
MTNR	melatonin receptor
mTOR	mammalian target of rapamycin
MUP	major urinary protein
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NAT	serotonin *N*-acetyltransferase
NCBI	National Center for Biotechnology Information
NCoR	nuclear receptor co-repressor
NDF	neu differentiation factors
NEB	negative energy balance
NEFA	non-esterified fatty acid
NF-κB	nuclear factor-κB
NF-Y	nuclear factor-Y
NGF	nerve growth factor
NHEJ	non-homologous end-joining
NK	natural killer (cell)
NK1R	neurokinin 1 receptor
nNOS	neuronal NOS
NO	nitric oxide
NOEL	no observed effects limit
NOR-1	neuron-derived orphan receptor-1
NOS	nitric oxide synthase
NPY	neuropeptide Y

NR	nuclear receptor
NS	non-specific
NSAIDs	non-steroidal anti-inflammatory drugs
NURR1	nurr-related Factor-1
NTD	N-terminal domain
NTS	nucleus tractus solitaries (brain stem)
OATP-B	organic anion-transporting polypeptide B
OBPs	odorant-binding proteins
Ob-R	leptin receptor
OCs	ovocleidins
OCT4	octamer-binding transcription factor 4
OCXs	ovocalyxins
OECD	European Organization for Economic Cooperation and Development
o,p′-DDT	1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane
OPG	osteoprotegerin
OPN	osteopontin
OR	odour receptor
ORco	OR co-receptor
OSNs	olfactory sensory neurons
oST	ovine somatotrophin
OVT	ovotransferrin
OXM	oxyntomodulin
OXR	orexin receptor
p160 SRC	p160 steroid receptor co-activator
PAH	polycyclic aromatic hydrocarbon
PAM	protospacer-adjacent motif
PAMPs	pathogen-associated molecular patterns
PBAN	pheromone biosynthesis activating neuropeptide
PBPK	physiologically based pharmacokinetic (model)
PC	prohormone convertase
PCBs	polychlorinated biphenyls
PCPA	*p*-chlorophenylalanine
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDI	potential daily intake
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
PER	Period protein
PGCs	primordial germ cells
PGC1α	peroxisome proliferator-activated receptor-γ co-activator 1α
PGDPs	proglucagon-derived peptides
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PGRMC1	progesterone receptor membrane component 1
PI3K	phosphatidylinositol 3′ kinase
PIF	prolactin-inhibiting factor (prolactin release-inhibiting hormone, PIH)
PIP2	phosphatidylinositol-4,5-bisphosphate
PK	pyruvate kinase
PKA	protein kinase A
PKC	protein kinase C

PKCI	protein kinase C inhibitor
PLC	phospholipase C
PLGA	poly(d,l-lactide-co-glycolide)
PMA	phorbol 12-myristate 13 acetate (tetradecanoylphorbol acetate, TPA)
PMCA2	plasma membrane Ca^{2+} ATPase 2
PMSG	pregnant mare serum gonadotrophin (equine chorionic gonadotrophin, eCG)
PNR	photoreceptor NR
POA	preoptic area
POMC	pro-opiomelanocortin
PP	pancreatic polypeptide
PPAR	peroxisome proliferator-activated receptor
ppb	parts per billion
PPRE	peroxisome proliferator-activated receptor responsive element
PR	progesterone receptor
PRH (PRF)	prolactin-releasing hormone (vasoactive intestinal peptide, VIP)
PRID	progesterone-releasing intravaginal device
PRKAG3	protein kinase, AMP-activated gamma 3 subunit
PRL	prolactin
PRLR	prolactin receptor
PRRS	porcine reproductive and respiratory syndrome
PSE	pale, soft, exudative (meat)
PSS	porcine stress syndrome
pST	porcine somatotrophin
PT	promoter
PTH	parathyroid hormone, parathormone
PTH1R	parathyroid hormone receptor
PTHrP	parathyroid hormone-related peptide
PTP1B	protein tyrosine phosphatase 1B
PTU	6-propyl-2-thiouracil
PUFA	polyunsaturated fatty acid
PVN	paraventricular nucleus
PXR	pregnane X receptor
PYY	peptide YY (peptide tyrosine tyrosine)
QC	quality control
QMP	queen mandibular pheromone
QTL	quantitative trait locus
RA	retinoic acid
rAB	recombinant antibody
RALDH2	retinaldehyde dehydrogenase
RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
RAR	retinoic acid receptor
RE	responsive element
RELMs	resistin-like molecules
REM	rapid eye movement
RER	rough endoplasmic reticulum
REV-ERB	reverse-Erb receptor
RFLP	restriction fragment length polymorphism
RFRP3	RF-amide-related peptide-3, GnIH
RIA	radioimmunoassay
RNAi	RNA interference-mediated gene knockdown

ROR	retinoic acid-related receptor
ROR-1	tyrosine kinase-like orphan receptor-1
RSPO1	R-spondin 1
RSTK	receptor serine/threonine kinases
rT_3	reverse T_3
RU-486	Mifepristone
RXR	9-*cis* retinoic acid receptor
Ryr	ryanodine receptor (calcium release channel, CRC)
SA (SAM)	sympathetic nervous system activation of the adrenal medulla
SARM	selective androgen receptor modulator
SCD	stearoyl-CoA desaturase
SCFAs	short-chain fatty acids, VFAs
SCID	severe combined immunodeficiency (mice)
SCN	suprachiasmatic nuclei (of the hypothalamus)
SDS	sodium dodecyl sulfate
SELEX	systematic evolution of ligands by exponential enrichment
SERCA	sarco(endo)plasmic reticulum ATPase
SERM	selective oestrogen receptor modulator
SF-1	steroidogenic factor 1
sgRNA	single-guide RNA
SH2	src homology region 2
SHBG	sex hormone-binding globulin
SHP	small heterodimer partner
siRNA	small interfering RNA
SMAD, Smad	suppressor of mothers against decapentaplegia
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SN system	sympathoneuronal system, LUC-NE
SNP	single nucleotide polymorphism
SNMP1	sensory neuron membrane protein 1
SOCS3	suppressor of cytokine signalling 3
SOX9	SRY box
SPCA1/2	secretory plasma Ca^{2+} ATPases
SPF	specific-pathogen-free
SPLAT®	Specialized Pheromone and Lure Application Technology
SPPS	solid-phase peptide synthesis
SR	sarcoplasmic reticulum
SR	steroid receptor
SRC	steroid receptor co-activator
src	sarcoma (abbreviation)
SRD5A	steroid 5α-reductase enzyme
SREBP	sterol regulatory element-binding protein
SRP	signal recognition particle
SRY	sex-determining gene on Y
SS (STS)	somatostatin (growth hormone release-inhibiting hormone, GHIH)
SSRS	skin stress response system
ST	somatotrophin (growth hormone, GH)
StAR	steroid acute regulatory protein
STAT	signal transducer and activator of transcription
STS	steroid sulfatase
SULT1A1	phenol sulfotransferase
SUMU	small ubiquitin-like modifier protein

T	testosterone
T1AM	3-iodothyronamine
T_2	diiodothyronine
T_3	triiodothyronine
T_4	thyroxine
TAAR1	trace amine-associated receptor 1
TALEN	transcription activator-like effector nuclease
TAT	transactivator protein
TBA	trenbolone acetate
TBG	thyroid hormone-binding globulin
TBT	tributyltin
TCA	tricarboxylic acid (cycle)
TCD	thermal conductivity detector
TCDD	tetrachlorodibenzo-*p*-dioxin
TD	tibial dyschondroplasia
TF	transcription factor
Tf-Cr	transferrin-bound chromium
Tfm	testicular feminization (androgen insensitivity syndrome, AIS)
Tgb	thyroglobulin
TGF	transforming growth factor
TGR5	G protein-coupled bile acid receptor (GP-BAR1)
Th	helper T cells
THC	tetrahydrocannabinol
TLR4	toll-like receptor 4
TMB	3,3′,5,5′-tetramethylbenzidine
TMD	transmembrane domain
TMT	2,5-dihydro-2,4,5-trimethylthiazole (fox odour)
TNF	tissue necrosis factor
TP-1	trophoblast protein-1 (interferon-τ, IFN-τ)
TPA	tetradecanoylphorbol acetate (phorbol 12-myristate 13 acetate, PMA)
TPO	tyrosine peroxidase
TR	thyroid hormone receptor
tracrRNA	trans-encoded small RNA
TRH	thyrotrophin-releasing hormone
TRPC	transient receptor potential channel
TRPV	transient receptor potential vanilloid channel
TSH	thyroid-stimulating hormone (thyrotrophin)
TT	tetanus toxin
TTFL	transcription–translation feedback loop
TTR	transthyretin
TX	thromboxane
UCP	uncoupling protein
UFM	unilateral frequent milking
URO-EGF	urogastrone epidermal growth factor
USDA	United States Department of Agriculture
UTR	untranslated region
UVB	ultraviolet B
V1R and V2R	vomeronasal organ receptors
VDR	1,25-hydroxyvitamin D_3 receptor
VEGF	vascular endothelial growth factor
VFAs	volatile fatty acids, SCFAs

VIP	vasoactive intestinal peptide (prolactin-releasing hormone, PIF)
VLDL	very-low-density lipoprotein
VMN	ventromedial nucleus
VNO	vomeronasal organ
VRE	vancomycin-resistant enterococci
WADA	World Anti-Doping Agency
WHO	World Health Organization
WNT	wingless-related integration
WSXWS	(a common sequence motif) Trp-Ser-X-Trp-Ser
XOS	xylo-oligosaccharides
ZFN	zinc finger nucleases
ZP	zona pellucida

1 Hormone and Receptor Structure and Function

This chapter covers the basic concepts of endocrine function, starting with how hormones function, how they are synthesized and released and details of their metabolism and clearance. The structure and function of receptors are then described, starting with the extracellular receptors; this includes the G protein-coupled receptors and their second messenger systems along with the catalytic receptors. Intracellular receptors are then covered, including details of their functional domains and interaction with chromatin. Finally, the integration of hormone action via the hypothalamic–pituitary axis is described.

1.1 Introduction

Key concepts

- Hormones are signalling molecules that modulate the activity of a target tissue.
- Hormones maintain homeostasis and also drive physiological processes.
- Hormone action is regulated by feedback (usually negative but sometimes positive).
- More than one hormone can interact to affect a biological response in different ways.
- Hormones affect gene expression, catalytic rates of enzymes and transport processes.
- Selectivity of hormone action is due to specific receptors in target cells or selective delivery to the target cells.
- The major structural groups of hormones are steroids, proteins and amino acid and fatty acid derivatives.

What is a hormone?

A hormone is a chemical messenger that coordinates the activities of different cells in a multicellular organism. In 1902 William Bayliss and Ernest Starling described the actions of secretin, a hormone produced by the duodenum to stimulate the flow of pancreatic juice (Bayliss and Starling, 1902). Starling would introduce the term 'hormone' for the first time during a lecture in 1905 (Starling, 1905). (For more historical information, see the texts by Hadley and Levine, 2006, and by Henderson, 2005.) The classical endocrine definition of a hormone is that it is synthesized by particular endocrine glands or scattered cells and then enters the bloodstream to be carried to a target tissue some distance away, which has specific receptors that bind the hormone, causing the cell to respond to the hormone by altering its metabolism. Other mechanisms of hormone delivery also exist (Fig. 1.1).

Neuroendocrine hormones are synthesized by nervous tissue and carried in the blood to the target tissue. An example of this is the various releasing and release-inhibiting hormones that are produced in the hypothalamus, which travel to the anterior pituitary via the hypothalamic–pituitary blood portal system (see Section 1.4). Neurocrine hormones are released into the synaptic cleft by neurons that are in contact with the target cells. Paracrine hormones diffuse to neighbouring cells, while autocrine hormones feed back on the cell of origin in a form of self-regulation. These mechanisms are important for the local regulation and coordination of cellular metabolism, as occurs in growth in hair follicles (see Section 4.3). At the other extreme, pheromones are produced by one animal and released into the environment to be received by other animals (see Section 6.2). Thus, hormones can best be considered as 'signalling molecules' that coordinate activities of cells, organisms and populations.

Why are hormones necessary?

Hormones are involved in maintaining homeostasis within an organism, which is a consistency of the

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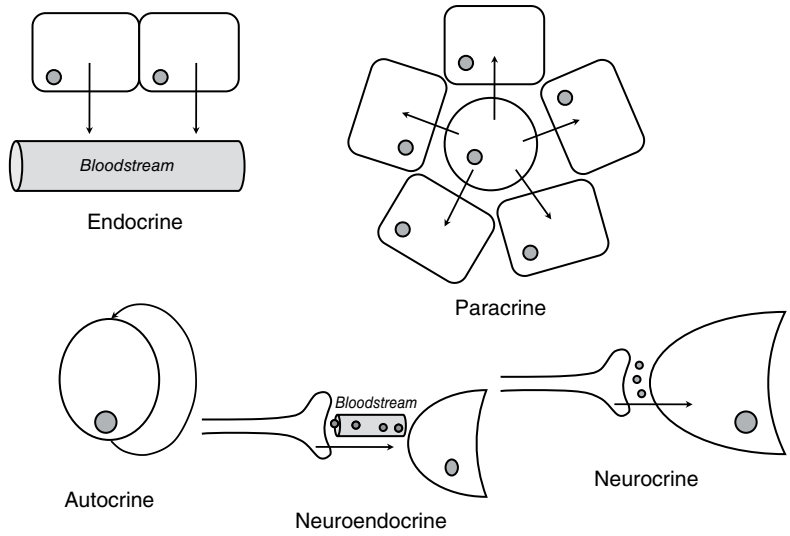


Fig. 1.1. Mechanisms of hormone delivery.

internal environment that is maintained for the benefit of the whole organism. Homeostasis was first recognized in the 19th century by Claude Bernard, who noted that the internal environment (i.e. the fluid bathing cells) had to be regulated independently of the external environment. Being able to regulate and maintain its internal environment gives the animal freedom from changes in the external environment, allowing it to live in changing or harsh environments, but there are metabolic costs associated with maintaining homeostasis. For example, maintenance of a constant body temperature allows animals to function in cold environments, while cold-blooded animals (poikilotherms) only function during warm temperatures. The added energy costs of maintaining deep-body temperature above that of the environment mean that warm-blooded animals have a higher energy requirement for maintenance than do poikilotherms.

However, hormones do more than maintain homeostasis. They also control and drive a variety of physiological and metabolic processes; they are involved in the response to external stimuli as occurs during the fight-or-flight response; and they drive cyclic and developmental programmes such as sex differentiation and ovulation.

Hormones are subjected to tight regulation by feedback control from target organs that consists of cyclic systems (loops) that control the amount of hormone released. Homeostasis is maintained by negative feedback while driven systems are under positive feedback control (Fig. 1.2; see also Figs 1.58 and 2.4 for specific examples). In negative feedback control, an endocrine tissue produces a hormone that affects the production of a metabolite by the target tissue. The metabolite then interacts with the endocrine gland to reduce the production of the hormone. This forms a cyclic system in which the metabolites are maintained at a particular level. For example, glucose homeostasis is maintained at 4–6 mM in blood by the pancreas, which produces insulin or glucagon in response to changes in levels of blood glucose. Increased blood glucose causes insulin release, which stimulates glucose uptake by adipose tissue and muscle, while decreased blood glucose causes glucagon release, which stimulates the release of glucose from storage. When glucose levels return to their homeostatic levels, the release of these hormones is decreased.

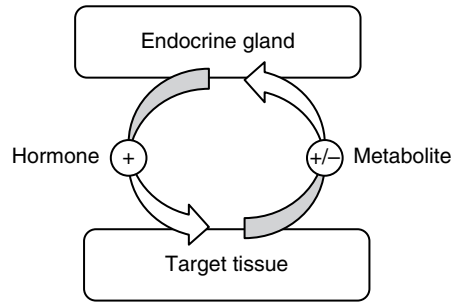


Fig. 1.2. Feedback system to regulate hormone production.

The set point of the system can also be altered to affect the levels of the metabolite by altering the sensitivity of the target tissue to the hormone or the sensitivity of the endocrine gland to negative feedback from the metabolite. This adjustment of the set-point of metabolic systems to maintain a physiological equilibrium is known as allostasis. This is important to allow the organism to adapt to a new or changing environment, such as coping with a sustained stressful situation (see Section 6.3).

When hormones are used to drive change in an organism, levels of hormone increase to some peak, and this occurs by positive feedback. Positive feedback amplifies the response, so the tissue must be desensitized or turned over to stop the response. An example of this response is the surge of luteinizing hormone (LH) that leads to ovulation (see Section 5.1). LH produced by the pituitary gland stimulates the developing ovarian follicle to produce oestrogen, which stimulates the hypothalamus to produce gonadotrophin-releasing hormone (GnRH) and increase LH production by the pituitary. This produces a surge of LH, which decreases only after the follicle ovulates to break the cycle (Fig. 1.3; also see Fig. 5.4).

How do hormones function?

Hormones cause a trigger effect to modulate the activity of the target tissue. The effects of hormones are seen long after levels of the hormone return to basal values. In contrast, nervous signals are short-lasting and more immediate. However, nervous signals can regulate hormone production as well, so there is a link between the endocrine and nervous systems called neuroendocrine transduction. Hormones are present in trace amounts in plasma, usually ranging from 10^{-9} to 10^{-6} g ml^{-1}. They are present at all times, in order to maintain receptors in the target tissue and keep the tissue primed for a response. The effect of a hormone depends on changes in the concentration of the hormone, and hormones are secreted in variable amounts according to need (for example, see Fig. 2.3). There is a constant turnover by inactivation and excretion of the hormone to return the levels of hormone back to basal levels. The amount of hormone response by target cells depends on the level of synthesis and release of the hormone, the level of biological activity of the hormone (i.e. whether it is present as an inactive precursor or bound to a carrier protein; see Section 1.2) and the rate of turnover and inactivation of the hormone.

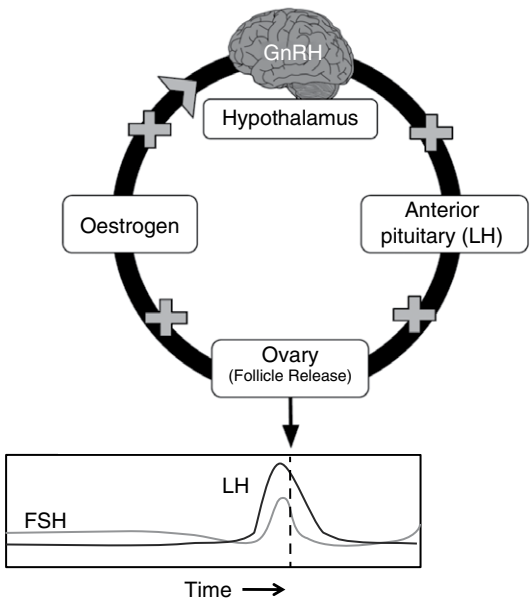


Fig. 1.3. Positive feedback system leading to the LH surge and ovulation.

The combined effects of more than one hormone on a biological response can occur in a number of different ways (Fig. 1.4). The actions of different hormones are concerted or additive if they cause the same response and the combined effect of the hormones is simply the sum of the actions of the individual hormones separately. This additive effect suggests that the two hormones act by different mechanisms to cause the response. In some cases, two hormones can cause the same response but the effects due to the different hormones are non-additive. This implies that the two hormones may act by the same common mechanism and the response was thus limited to a set maximum. The effects of two different hormones are synergistic when the combined effect of the two hormones together is more than the sum of the separate effects of the individual hormones. This suggests that the hormones interact to amplify the response of the individual hormones alone. Some hormones can also have antagonistic effects where one hormone reduces the response of a second hormone. For example, food intake (see Section 3.10) is regulated by both orexigenic hormones (neuropeptide Y (NPY) and agouti-related peptide (AgRP)), that increase feeding, and anorexigenic hormones (pro-opiomelanocorpin (POMC) and cocaine- and amphetamine-related transcript (CART)), that decrease feeding.

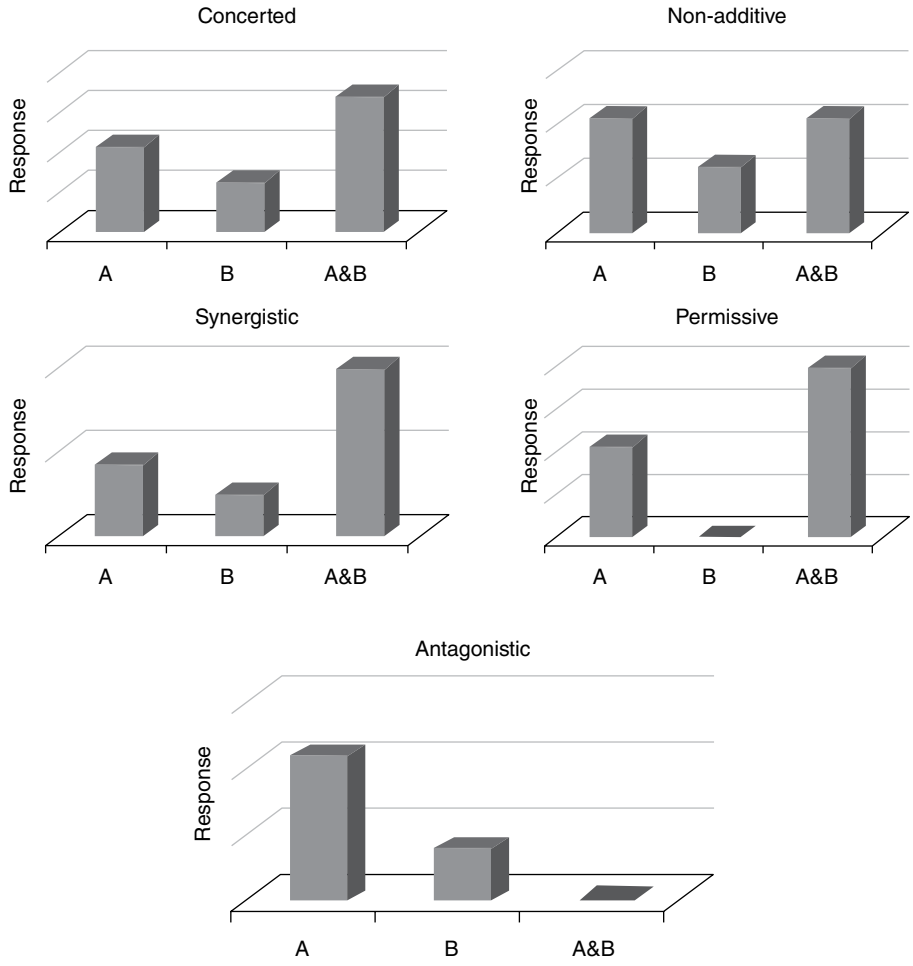


Fig. 1.4. Various actions of hormones.

Some hormones, for example steroid hormones and thyroid hormones, can have a permissive action and have no effect on their own but must be present for another hormone to have an effect. Permissive and synergistic effects of hormone could occur by increasing the number of receptors or affecting the activity of the second messenger system for the second hormone. For example, oestradiol has a permissive action for progesterone by inducing the expression of progesterone receptors in the oviduct (see Fig. 1.54).

What effects are due to hormones?

Hormones cause changes in cellular metabolism but they do not make a cell do something it was not previously capable of doing. Hormones do not directly cause changes in gene structure but can bind to receptors and activate genes to influence gene expression and ultimately protein synthesis. This can occur through changes in gene transcription to produce mRNA, effects on RNA stability or changes in mRNA translation to produce protein, and effects on protein stability. Hormones can alter catalytic rates of enzymes and other proteins, by mechanisms such as the phosphorylation and dephosphorylation of proteins, which are transient modifications of proteins that alter their structure and function. Hormones can also alter cellular transport and membrane permeability to affect active transport processes, ion movements, intracellular trafficking, exocrine secretion and water permeability.

These general biochemical mechanisms of hormones can cause a variety of physiological effects in the animal. Hormones can:

- cause morphological changes, such as the differences in body shape between males and females;
- act as mitogens to accelerate cell division or alter gene expression to trigger differentiation of cells (e.g. insulin-like growth factor-1 (IGF-1));
- stimulate the overall rate of protein synthesis or the synthesis of specific proteins;
- be involved in stimulating smooth muscle contraction (for example, oxytocin stimulates contraction of the myoepithelium in the mammary gland for milk ejection);
- affect exocrine secretions (for example, secretin, a peptide hormone from intestinal mucosa, stimulates pancreatic secretions);
- control endocrine secretions, and a number of trophic hormones from the anterior pituitary can stimulate or inhibit hormone secretion from target organs;
- regulate ion movements across membranes and control permeability to water (for example, antidiuretic hormone (ADH, vasopressin) increases water reabsorption by kidney); and
- have a dramatic effect on behaviour, such as sex-related behavioural characteristics, maternal behaviour, nesting activity and broodiness (see Chapter 6).

How is hormone action selective?

The method of hormone delivery to the target cells and the presence of specific receptors in the target cells can determine the selectivity of hormone action. For example, the hypophyseal–portal blood system linking the hypothalamus to the pituitary gland delivers releasing and release-inhibiting hormones from the hypothalamus directly to the target cells in the anterior pituitary. Smaller quantities of hormone are needed in this system, since there is less dilution of the hormone in selective delivery systems compared with hormones that reach their target via the peripheral circulation. Many hormones, particularly lipophilic hormones, are linked to carrier proteins in the blood, which stabilize the hormone and increase its half-life in the circulation. For example, sex hormone-binding globulin is synthesized in the liver and binds testosterone and oestradiol in the circulation with a high affinity. However, the main factor that determines the sensitivity of a particular tissue to a hormone is whether or not the tissue contains the specific receptor for the hormone – the tissue will not respond to the hormone unless it has enough of the specific receptor for the hormone. This is important for the hormone to function in a target tissue but is also necessary for the feedback regulation of the production of a hormone by the endocrine tissue. A hormone can sometimes bind to a number of different receptor subtypes and these may be differentially expressed in various tissues and cells, resulting in different tissue-specific effects of hormones.

Receptors are specific proteins present in target cells that bind a particular hormone with high affinity and initiate a response. Receptors are normally present in small numbers (10,000 molecules per cell). There are two general types: cell surface receptors (Fig. 1.5) and intracellular receptors (Fig. 1.6). Peptide and protein hormones are polar molecules that generally cannot pass through the lipids in the cell membranes and do not enter the cell but interact with receptors on the cell surface. For some cell-surface receptors, a second messenger system is needed to transmit the hormone response signal from the outside to the inside of the cell. Binding to the receptor results in the activation of a protein kinase, which phosphorylates specific proteins within the cell to alter their function. Steroid hormones and thyroid hormones can diffuse through the cell membrane to enter the cell and interact with intracellular receptors and regulate gene expression.

Types of hormones

The major structural groups of hormones are as follows.

- Steroids. These are produced by stepwise conversion of cholesterol by a series of enzymes, are lipid soluble and are produced and secreted as needed.
- Proteins, polypeptides and glycoproteins. These are the products of genes, accumulate in Golgi vesicles, are secreted by exocytosis and are water soluble.
- Amino acid derivatives (especially derivatives of tyrosine and tryptophan).
- Fatty acids and derivatives, eicosanoids such as prostaglandins. These are produced locally, are derived from cell membrane phospholipids

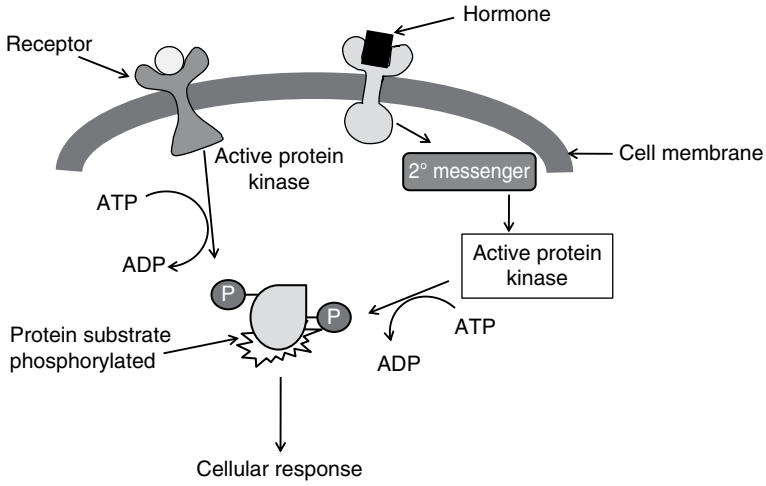


Fig. 1.5. Action of hormones via cell surface receptors.

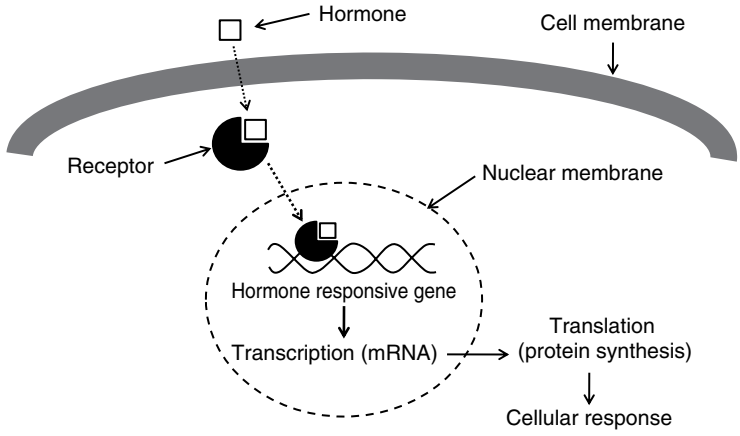


Fig. 1.6. Action of hormones via intracellular receptors.

(arachidonic acid) and have mainly autocrine and paracrine effects.

The structures of some non-protein hormones are given in Fig. 1.7.

Location of endocrine glands

The location of the key endocrine glands is given in Fig. 1.8. Table 1.1 lists the hormones produced by these glands and their function. Applications involving many of these hormones are covered in this text and the relevant sections are listed in Table 1.1.

1.2 Synthesis, Release and Metabolism of Hormones

Key concepts

- Protein hormones are produced by gene transcription and translation.
- Signal peptides direct proteins to various cellular compartments or export from the cell.
- Some peptide hormones are synthesized as a larger inactive precursor, a prohormone.
- Steroid hormones are produced from cholesterol by a series of reactions that modify the functional groups on the common steroid nucleus.

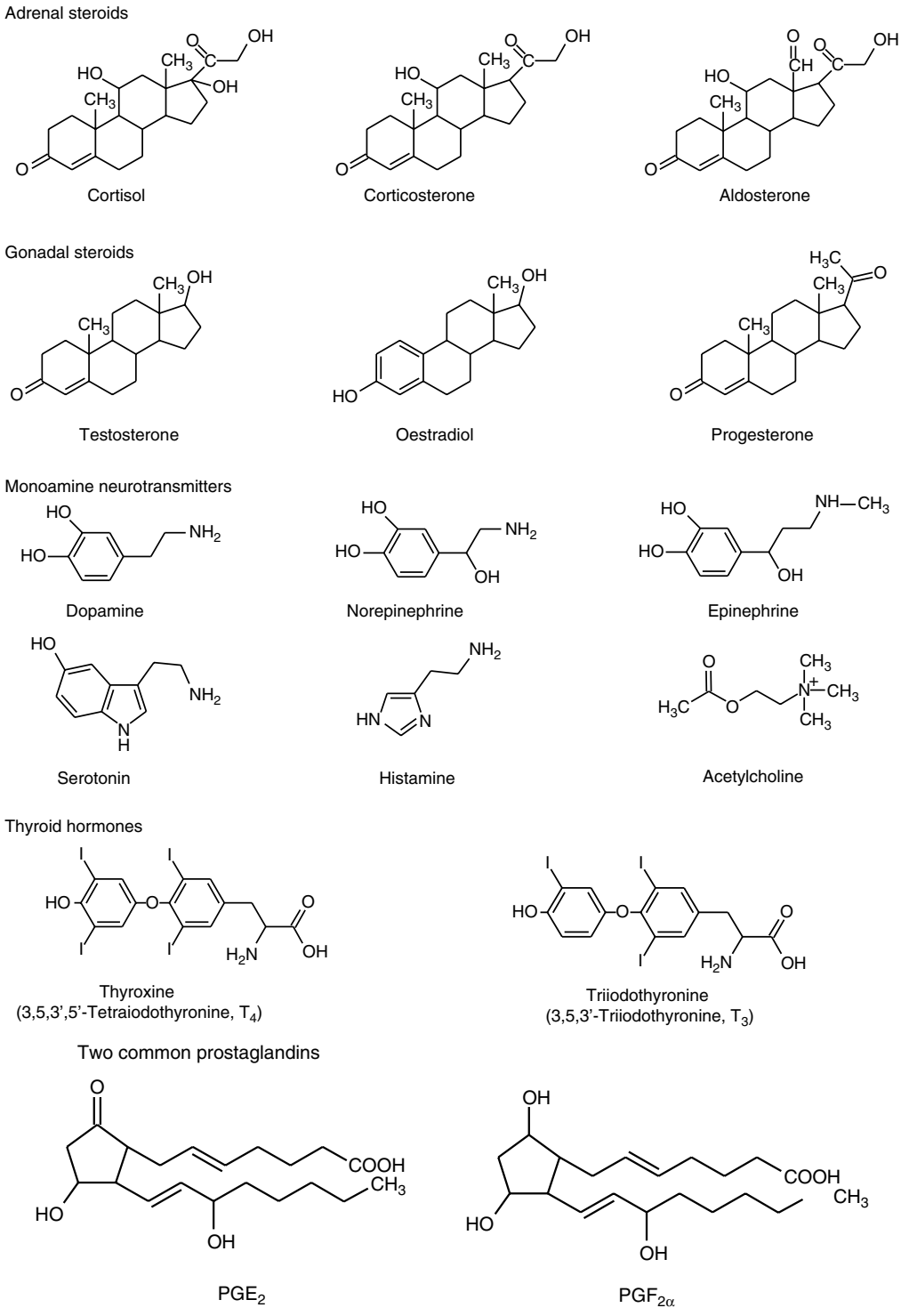


Fig. 1.7. Structures of representative non-protein hormones.

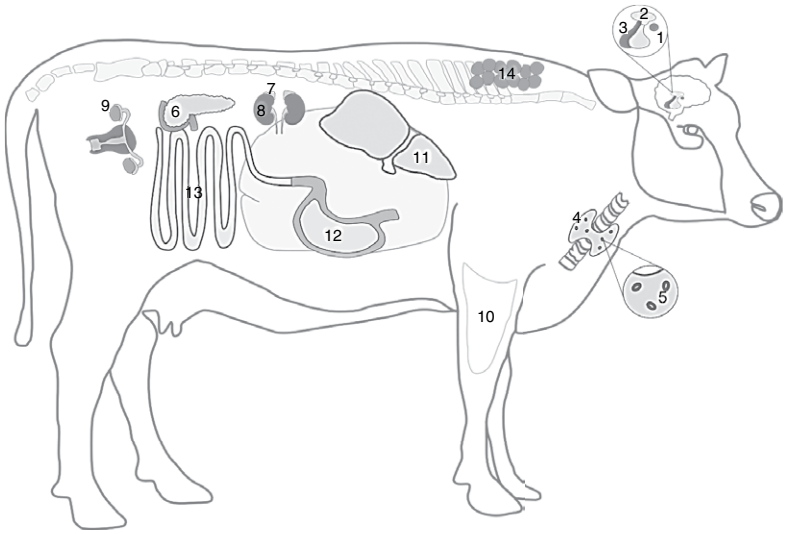


Fig. 1.8. The location of key endocrine glands in cattle.

- Eicosanoids are produced from fatty acids in phospholipids within cell membranes.
- Thyroid hormones are produced by iodination of tyrosine residues in thyroglobulin.
- Hormones are released in response to trophic hormones, neuroendocrine transduction or stimulus–response coupling.
- Lipophilic hormones diffuse out of the endocrine cells after synthesis and circulate associated with carrier proteins.
- Protein hormones are packaged in vesicles and secreted by exocytosis in response to various stimuli.
- Peptide hormones are degraded by peptidases in the target tissue and other tissues.
- Steroid hormones are metabolized in the liver by a two-stage process that makes them more water soluble.
- Steroids may also be stored as sulfoconjugates.

Synthesis of protein hormones

Peptide and protein hormones consist of a linear chain of amino acids. As with any protein, the specific sequence of the different amino acids in the protein determines the primary structure and the properties of the protein. The amino acid sequence information for a protein is contained in the sequence of bases (A,C,G,T) in the coding regions (exons) of the gene that codes for the protein. A three-base sequence (codon) codes for one amino acid; this is known as the genetic code. This code is copied from DNA into RNA by transcription; the RNA is processed into messenger RNA (mRNA), and the mRNA is used to direct protein synthesis on ribosomes by the process of translation.

Protein hormones are water soluble and therefore cannot diffuse out of cells. Instead, they are packaged in vesicles and released by exocytosis. This process is directed in part by signal peptides, which are short sequences of 15–30 hydrophobic amino acids located at the amino terminal (beginning) of proteins. The presence of a signal sequence (S) directs the newly synthesized protein into the endoplasmic reticulum and from there to export from the cell. Other proteins enter the cytosol and from there are directed to the mitochondria (M) or nucleus (N) or other sites within the cell. Proteins move between the various compartments by vesicular transport. The uptake of proteins by particular vesicles is controlled by the sorting signal sequences in the proteins (Fig. 1.9). The program SignalP 5.0 can be used to identify signal peptides and their cleavage sites and the program DeepLoc-1.0 predicts the subcellular localization of eukaryotic proteins. These programs can be accessed at https://services.healthtech.dtu.dk/. For more information, see the review by Nielsen *et al.* (2019).

Table 1.1. Summary of hormones produced by various endocrine glands and their function.

Endocrine glands	Hormones produced	Physiological response	Relevant book sections
Hypothalamus	TRH	TSH and PRL by anterior pituitary	1.4
	GnRH and GnIH	LH and FSH	1.4, 4.2, 5.1, 5.2
	CRH	ACTH, β-endorphin, stress	1.4, 6.3
	GHRH and GHIH	GH	1.4, 3.4
	PRF (VIP) and PIH	PRL	1.4, 6.1
	MRF and MIH	MSH	1.4, 3.10
	NPY, AgRP	Increased feed intake	3.10
	Orexin, MCH	Increased feed intake	3.10
	POMC, CART	Decreased feed intake	3.10
Anterior pituitary (adenohypophysis)	GH	Somatomedin by liver	3.4
	PRL	Mammary gland/lactogenesis, broodiness	4.1
	TSH	Thyroid hormone	6.1
	FSH	E_2, follicular growth/ spermatogenesis	3.6
	LH	E_2 and P_4, ovulation/androgen synthesis	4.2, 5.1
	ACTH	Adrenal steroids	4.2, 5.1
	MSH	Melanogenesis, appetite	3.12, 6.3
	β-Endorphin	Analgesic	3.10
Posterior pituitary (neurohypophysis)	Oxytocin	Milk ejection	1.4
	Vasopressin	Antidiuretic hormone	1.4
Pineal	Melatonin	Seasonality, gonad function	5.1
Parathyroid	PTH	Calcium and phosphorus metabolism	4.1, 4.2
Thyroid	T_4 and T_3	Metabolic rate	3.6
Adrenal cortex	Corticosteroids	Carbohydrate metabolism	3.12, 6.3
	Aldosterone	Sodium retention	6.4
Adrenal medulla	(Nor)epinephrine	Alarm reactions	3.7, 6.3
Gonads	Androgens, oestrogens	Sexual development/behaviour	3.2, 3.3,
	Progestins	Pregnancy	4.1, 5.1, 5.2
	Inhibin, activin	FSH release	
	Relaxin/oxytocin	Parturition/milk let-down	
Pancreas	Insulin	Decreased blood glucose	3.8
	Glucagon	Increased blood glucose	
Gastrointestinal tract	Gastrin, GIP, secretin	HCl and bicarbonate	3.10, 3.4
	Motilin	Gastric activity	3.10
	Ghrelin	Increased feed intake	
	CCK, GLP-1, OXM, PYY, PP	Decreased feed intake	
Kidney	Erythropoietin	Blood cell formation	
Adipose	Leptin	Decrease appetite, increase energy utilization	3.5
	Adiponectin	Increase appetite, inhibit energy expenditure	3.5
	resistin	Antagonizes the effects of insulin	
	irisin	Browning of white adipose tissue (potentially)	
Various tissues	Eicosanoids	Smooth muscle	1.1, 3.9
	Growth factors	Growth and differentiation	4.1, 4.3

Newly synthesized protein hormones containing signal sequences are known as prehormones. Some peptide hormones are synthesized as part of a larger inactive precursor, called a prohormone. Examples of prohormones include proparathyroid hormone, the precursor of parathyroid hormone, and proinsulin, which is the precursor of insulin. Pro-opiomelanocortin (POMC) is the precursor of several trophic peptide hormones produced in the anterior pituitary (Fig. 1.10). These peptides are released by proteolytic cleavage of POMC by the prohormone convertase enzyme (PC1/3) and are further processed in the Golgi and secretory granules. The newly synthesized prohormone with a signal peptide is known as a preprohormone (Fig. 1.11).

The signal sequence of the secretory protein being synthesized is recognized by a cytosolic protein complex, the signal recognition particle (SRP), which guides the mRNA–ribosome complex to an SRP receptor in the membrane of the endoplasmic reticulum (ER). When it arrives at the ER, the signal sequence is transferred to the translocon, a protein-conducting channel in the membrane that allows the newly synthesized polypeptide to be translocated to the ER lumen. The signal sequence is then removed and the secretory protein is released into the ER lumen. Some proteins are glycosylated, which involves attaching sugar residues to asparagine, serine and other amino acid side chains to form glycoproteins. These sugar units can form complex branched chains and are a permanent post-secondary modification of the protein structure. Proteins are transported from the ER to the Golgi network, where they are packaged into secretory vesicles and the active hormone is generated by cleavage of the prohormone sequences. The secretory granules fuse with the plasma membrane to release their contents by exocytosis when the cell is stimulated.

A number of hormones, including the pituitary hormones luteinizing hormone (LH), follicle-stimulating

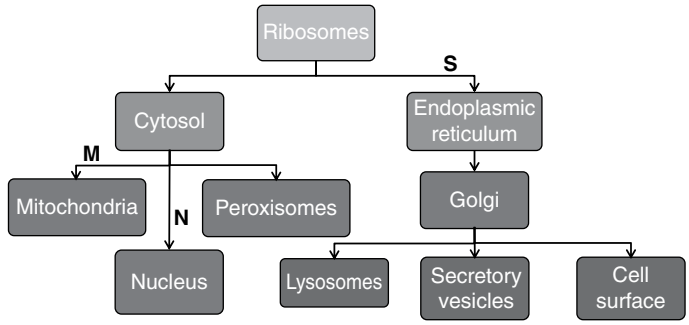


Fig. 1.9. Role of signal peptides in directing the movement of proteins within cells. A typical signal sequence (S) is: M-M-S-F-V-S-L-L-L-V-G-I-L-F-W-A-T-E-A-E-Q-L-T-K-C-E-V-F-Q- (a patch of hydrophobic amino acids is underlined). The typical signal (M) for importing into the mitochondria is: M-L-S-L-R-Q-S-I-R-F-F-K-R-A-T-R-T-L-C-S-S-R-Y-L-L-. The typical signal (N) for importing into the nucleus is: P-P-K-K-K-R-K-V-.

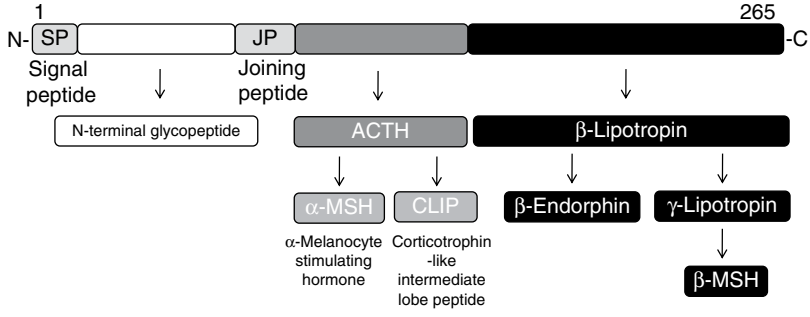


Fig. 1.10. Products of the pro-opiomelanocortin (POMC) gene.

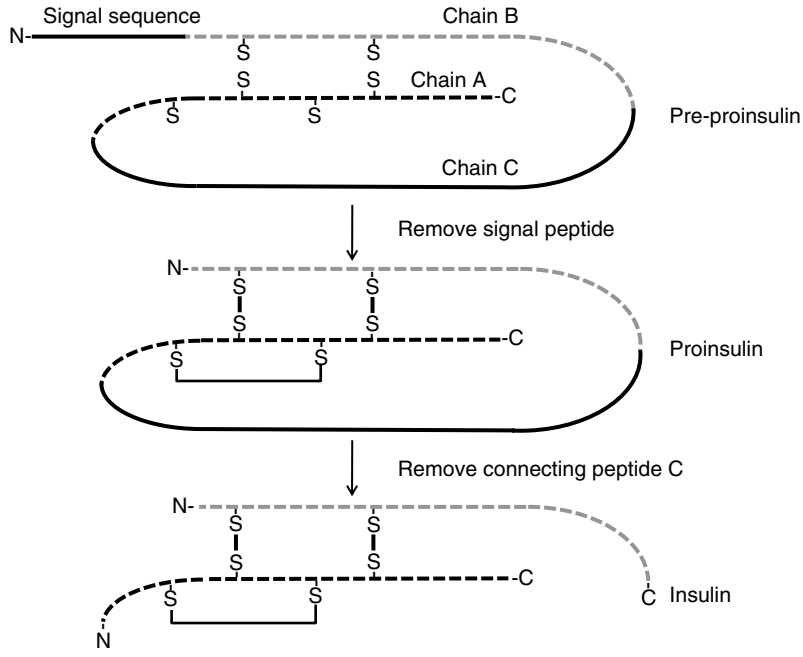


Fig. 1.11. Structure of insulin, illustrating the signal peptide and pro-sequence.

hormone (FSH) and thyroid-stimulating hormone (TSH), are glycoproteins. Some hormones also comprise multiple subunits, where each subunit is a different protein. These subunits are linked by disulfide bonds during packaging. For example, LH, FSH and TSH all share a common α subunit, but have individual specific β subunits (LHβ, FSHβ, TSHβ).

For more information on the mechanisms of protein secretion, see the reviews by Benham (2012) and Kim *et al.* (2018).

Synthesis of steroid hormones

Steroid hormones are produced in the gonads and in the cortex of the adrenal gland. The gonadal steroids include the progestins, oestrogens and androgens. Progesterone is a major progestin, which prepares the lining of the uterus for implanting of the ovum and is involved in the maintenance of pregnancy. Oestrogens, such as oestradiol, are involved in the development of female secondary sex characteristics and in the ovarian cycle. The androgens, such as testosterone, are involved in the development of male secondary sex characteristics. The adrenal cortex produces glucocorticoids and mineralocorticoids. Cortisol and corticosterone, major glucocorticoids in mammals and poultry, respectively, promote gluconeogenesis and fat and protein degradation. Aldosterone, a major mineralocorticoid, increases absorption of sodium, chloride and bicarbonate by the kidney to increase blood volume and blood pressure.

The synthesis of steroid hormones occurs on the smooth endoplasmic reticulum and in the adrenal mitochondria. Cholesterol is the precursor of all steroid hormones and is present as low-density lipoprotein (LDL) in plasma. Many of the steps in the biosynthesis of steroids involve an electron transport chain in which cytochrome P450 is the terminal electron acceptor and carries out hydroxylation reactions. The overall scheme is shown in Fig. 1.12.

The conversion of cholesterol to pregnenolone (Fig. 1.13) involves removal of the C6 side chain from cholesterol by hydroxylation at C20 and C22 and cleavage of this bond by the desmolase enzyme (cytochrome P450 side-chain cleavage (CYP11A1)). This step occurs in adrenal mitochondria and is stimulated by adrenocorticotrophic hormone (ACTH).

Pregnenolone is then converted to progesterone by oxidation of the 3-hydroxy to a 3-keto group and isomerization of the Δ5 double bond to a Δ4 double bond. Progesterone is converted to cortisol

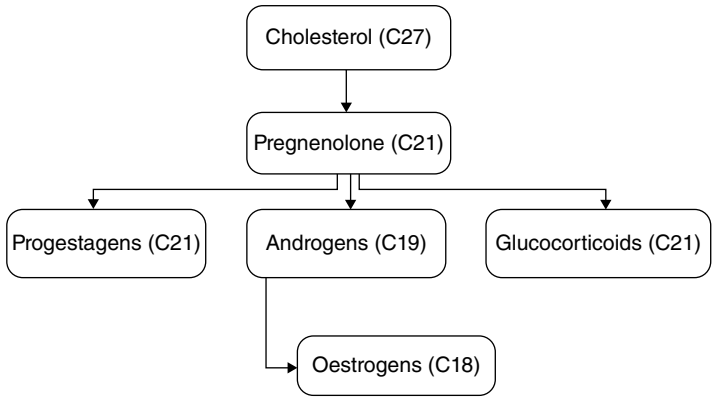


Fig. 1.12. Overall pathways of steroid hormone synthesis.

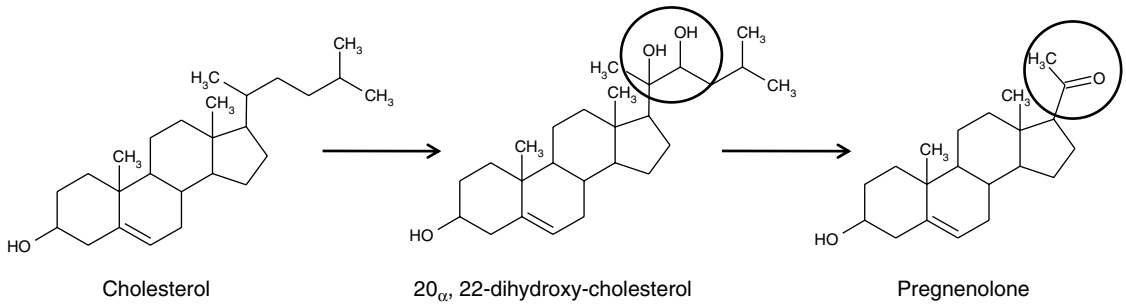


Fig. 1.13. Conversion of cholesterol to pregnenolone. The areas of the molecule that change are circled.

by hydroxylation at C17, C21 and C11. Progesterone is converted to aldosterone by hydroxylation at C21 and C11, and oxidation of the C18 methyl to an aldehyde (Fig. 1.14).

Progesterone is converted into androgens by hydroxylation at C17 and cleavage of the C17,20 bond by CYP17A1 to form androstenedione (an androgen). The 17-keto group is then reduced to a hydroxyl to form testosterone. Androgens are converted into oestrogens by loss of the C19 methyl group and aromatization of the A ring. Aromatization makes the A ring flat, which is a major structural feature of oestrogens. The formation of oestrogens from androgens is catalysed by the aromatase enzyme (CYP19A1) (Fig. 1.15).

Synthesis of eicosanoids

The eicosanoid hormones include prostaglandins, prostacyclins, thromboxanes and leukotrienes. They are produced locally within cell membranes and have autocrine and paracrine effects. They stimulate inflammation, regulate blood flow and blood pressure, affect ion transport and modulate synaptic transmission. They are synthesized from 20-carbon fatty acids, such as arachidonic acid (20:4), derived from membrane phospholipids (Fig. 1.16).

The enzyme cyclo-oxygenase (COX) catalyses the first step in the conversion of arachidonate to prostaglandins and thromboxanes. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and acetaminophen, inhibit COX and reduce the production of prostaglandins and thromboxanes. Prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) control vascular smooth muscle activity. Prostaglandin I_2 (PGI_2) is produced by the blood vessel wall and is the most potent natural inhibitor of blood platelet aggregation. Thromboxanes such as TXA_2 are produced by thrombocytes (platelets) and are involved in the formation of blood clots and the regulation of blood flow to the clot. Leukotrienes are made by leukocytes and are extremely potent in causing vasocontraction and inducing vascular permeability.

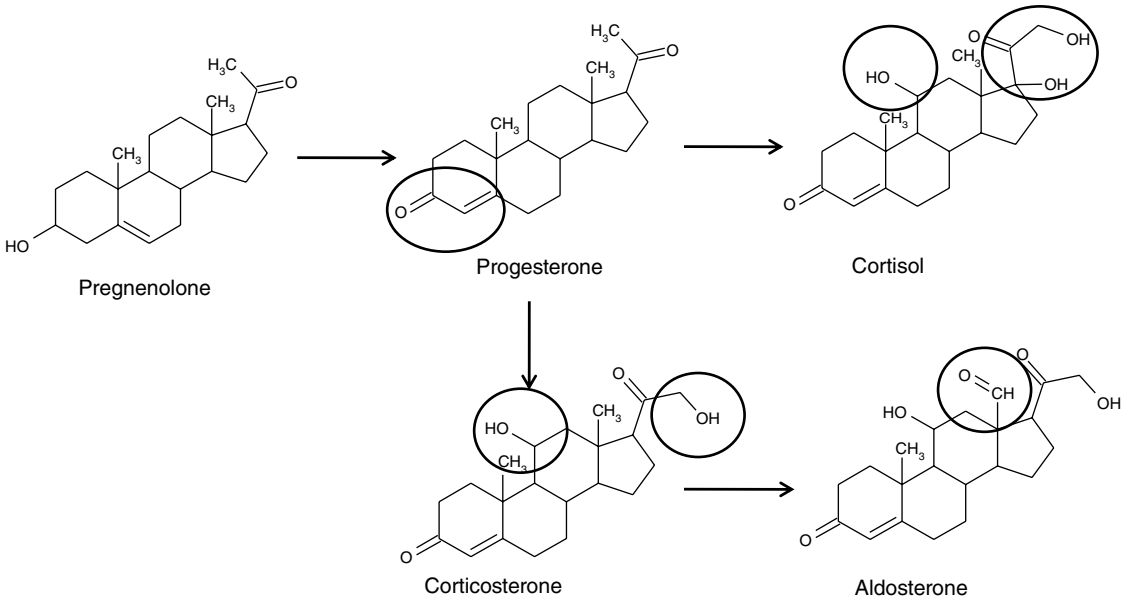


Fig. 1.14. Metabolism of pregnenolone to aldosterone. The areas of the molecule that change are circled.

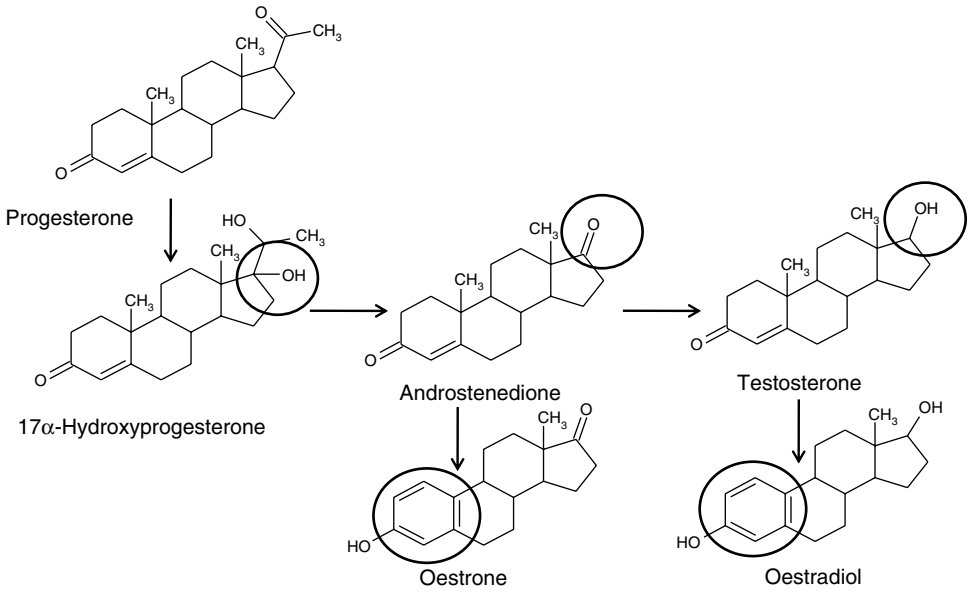


Fig. 1.15. Metabolism of progesterone to androgens and oestrogens. The areas of the molecule that change are circled.

Synthesis of thyroid hormones

The synthesis of thyroid hormones occurs in the follicles of the thyroid gland (Fig. 1.17) and is stimulated by TSH released from the anterior pituitary. TSH is released in response to thyrotrophin-releasing hormone (TRH) produced by the hypothalamus. Thyroid hormones are synthesized by iodination of tyrosine residues in the thyroglobulin protein

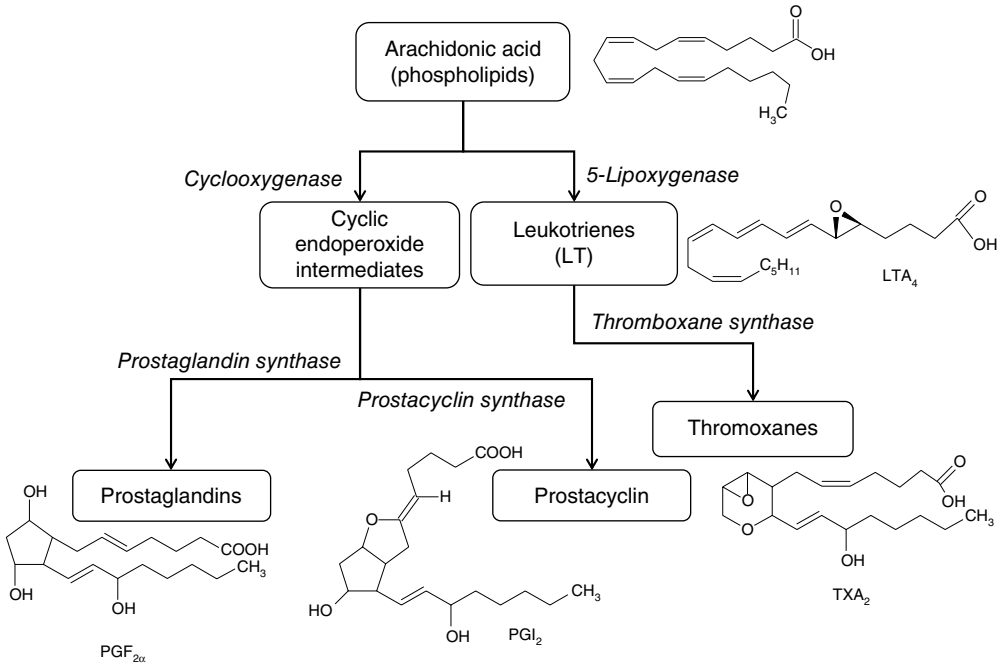


Fig. 1.16. Synthesis of eicosanoids.

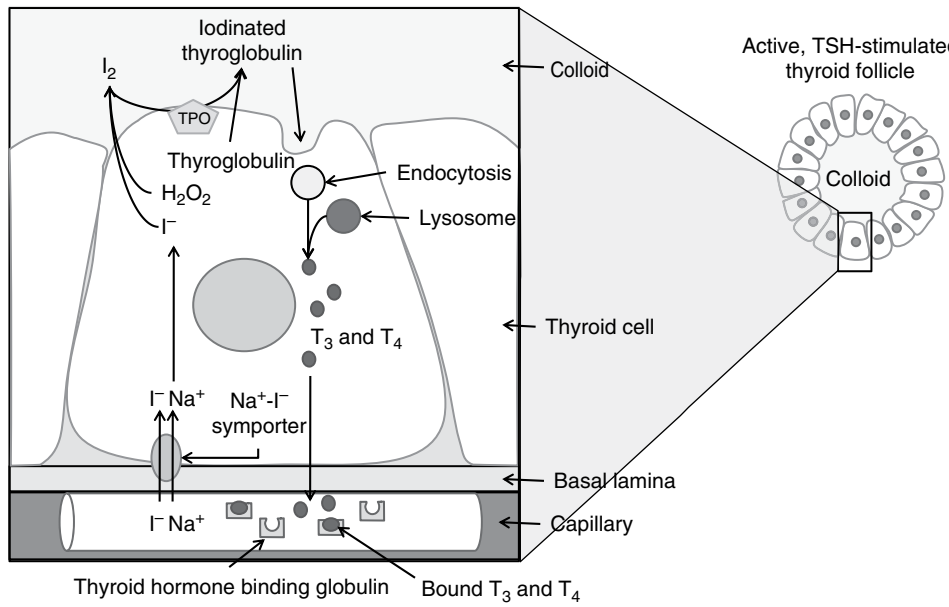


Fig. 1.17. The biosynthesis of thyroid hormones in follicular cells.

(Tgb), a glycoprotein with over 120 tyrosine residues. The process involves the active uptake of iodine from the blood through the follicular cells by an I^-/Na^+ symport transporter. The iodide then diffuses through the follicular cell and accumulates in the colloid, where it is used for iodination of thyroglobulin by thyroperoxidase (TPO). The iodinated thyroglobulin is taken up by endocytosis and

fused to lysosomes, where proteases degrade the thyroglobulin to release triiodothyronine (T_3) and thyroxine (T_4) (Fig. 1.18).

After cleavage in the follicular cell, T_3 and T_4 represent 10% and 90% of thyroid hormones, respectively. Thyroid hormones are lipophilic and diffuse across the basal membrane into the interstitial space and then into blood capillaries. In the blood, the thyroid hormones bind to carrier proteins, thyroid hormone-binding globulin (TBG), transthyretin (TTR) and albumin. T_4 is deiodinated to the active form T_3 in target tissues by the enzyme deiodinase (DIO2) (see Section 3.6).

Synthesis of monoamines

The monoamines are neurotransmitters and hormones derived from amino acids. The modification of tyrosine leads to synthesis of the catecholamines: dopamine, norepinephrine and epinephrine (also known as noradrenaline and adrenaline). The tyrosine comes from the diet or by conversion of phenylalanine in the liver. Tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, and L-DOPA is decarboxylated to dopamine (Fig. 1.19). Norepinephrine is produced by a second hydroxylation reaction, and then methylation of the amino group of norepinephrine yields epinephrine.

The adrenal medulla secretes the catecholamines epinephrine and norepinephrine; the ratio depends on the species and is 4:1 in humans. Secretion is partly under the control of the preganglionic sympathetic nerves that release acetylcholine. Acetylcholine depolarizes cells in the medulla, which induces Ca^{2+} entry and exocytosis of hormones. The effects are slower but five to ten times longer lasting than the sympathetic nervous system.

Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter derived from tryptophan and is found mainly in the gastrointestinal tract, with smaller amounts in the platelets and central nervous system. Histamine, released by basophils and mast cells, is a potent vasodilator and bronchial

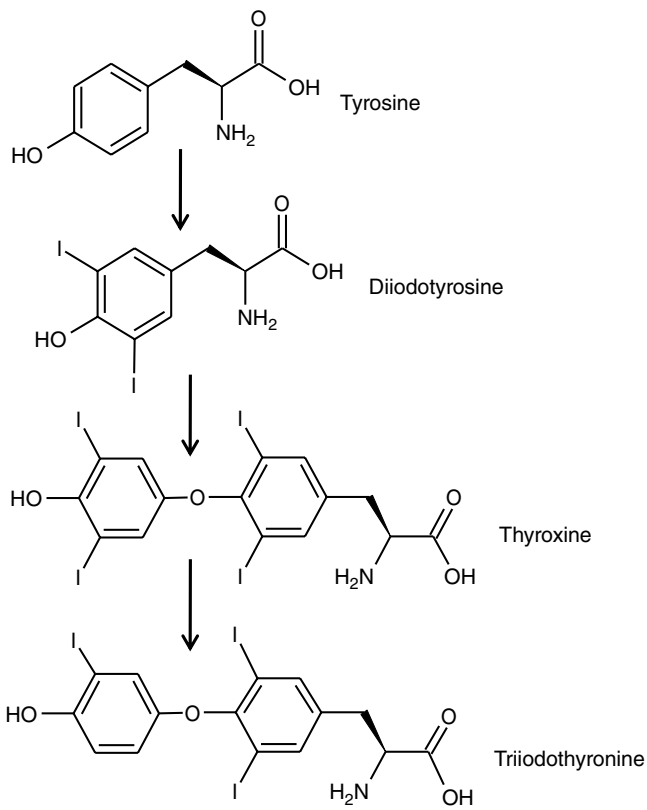


Fig. 1.18. The synthesis of thyroid hormones from tyrosine.

constrictor that is derived from decarboxylation of L-histidine. Acetylcholine, the chief neurotransmitter of the parasympathetic nervous system, is produced from acetylation of choline (see Fig. 1.7)

Hormone release

The mechanisms of release of hormones are directly related to their structure. Lipophilic hormones (steroid and thyroid hormones) are not stored after synthesis and diffuse out of the endocrine cells. They are insoluble in water and thus circulate associated with carrier proteins that are produced in the liver (Fig. 1.20). There are specific carriers, such as thyroid hormone-binding globulin and steroid-binding globulin, and non-specific carriers, such as albumin and prealbumin. The carrier proteins are large molecules that bind hormones and keep them

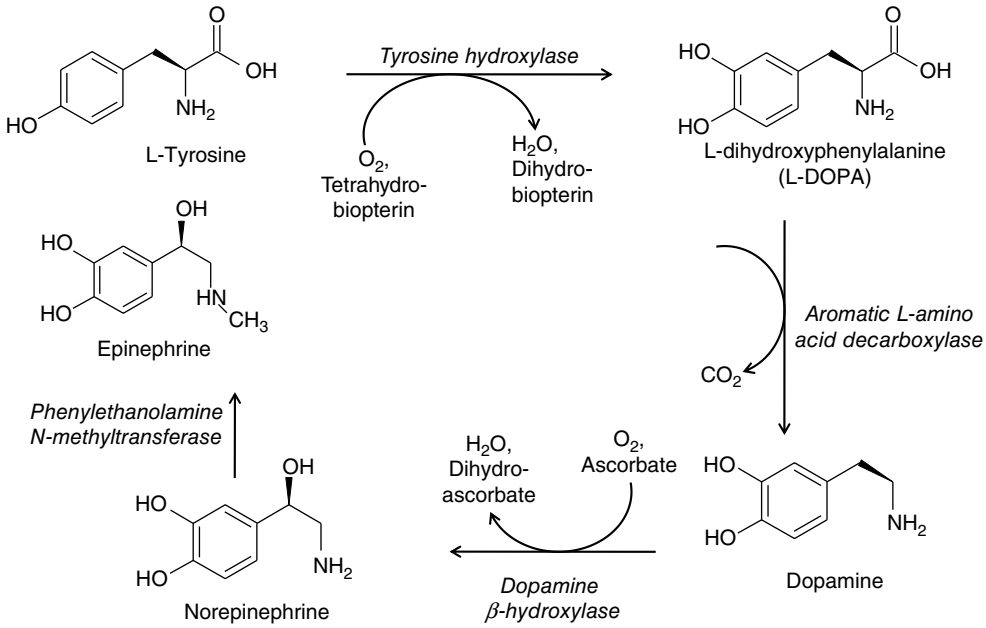


Fig. 1.19. Synthesis of monoamines.

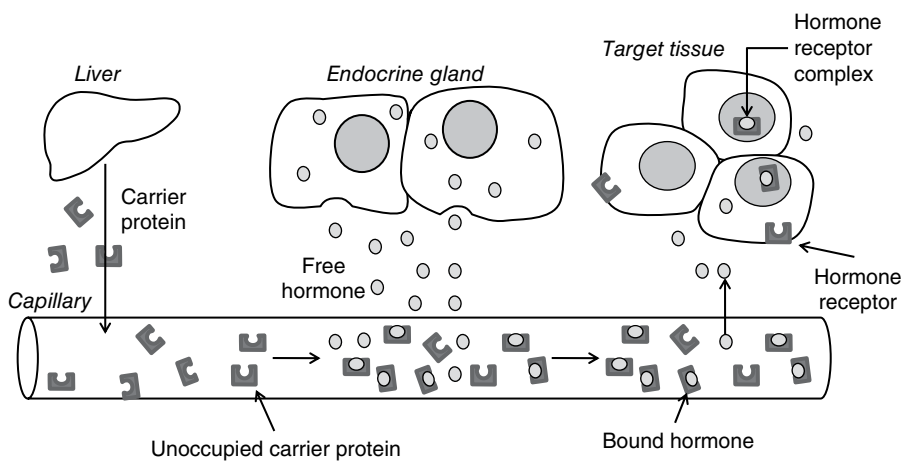


Fig. 1.20. Role of carrier proteins for hormones.

in the blood vessel, with only 5–10% of the hormone present in the free or unbound form that diffuses to tissues. This is the active portion, which is also susceptible to degradation and is involved in feedback control. Binding to protein carriers thus increases the half-life of steroids, and carrier proteins act as a hormone reservoir or buffer, protecting the hormone from degradation. Since the amount of active free hormone is also dependent on the concentration of its carrier protein, this provides an additional control mechanism regulating hormone activity (Fig. 1.21). For more information, see the review by Hammond (2016).

Water-soluble hormones (proteins and catecholamines) are polar molecules that cannot pass through the phospholipid membrane barrier of cells, so they are packaged in vesicles and secreted by exocytosis in response to various stimuli. They generally circulate free, except for insulin-like growth factor 1 (IGF-1), which circulates bound to a specific carrier protein. Both the synthesis and secretion of these hormones are regulated; this includes control at the level of gene transcription and translation, intracellular trafficking and exocytosis.

There are three main mechanisms regulating hormone release.

- Trophic hormones can stimulate hormone release; for example, TSH stimulates the release of thyroxine. The trophic hormones FSH and LH stimulate the synthesis and release of gonadal steroids, while ACTH stimulates the synthesis and release of adrenal steroids (see Section 1.4, Fig. 1.59).
- Hormones can be released in response to nervous stimuli from environmental cues such as light, smell, sound and temperature. This neuroendocrine transduction illustrates the integration of the nervous and endocrine systems. For example, a dark environment stimulates release of melatonin from the pineal gland.
- Hormones are also released in response to stimulus–response coupling, by sensing levels of various metabolites. For example, intracellular glucose levels control glucagon and insulin secretion (see Fig. 2.4), amino acids stimulate somatotrophin release and increase uptake of amino acids, while extracellular Ca^{2+} regulates parathyroid hormone and calcitonin-secreting cells.

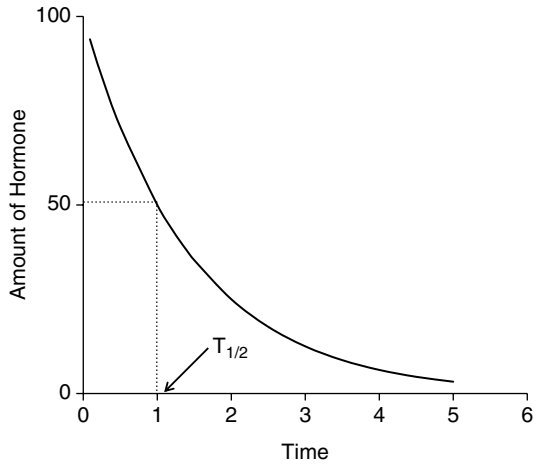


Fig. 1.21. Inactivation of hormones follows exponential decay.

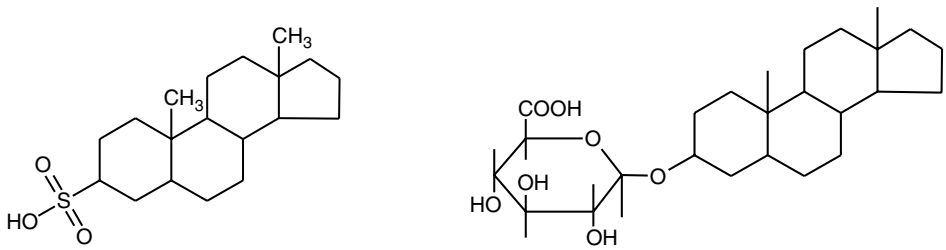
Metabolism and excretion of hormones

Hormones must be rapidly metabolized and removed so that feedback mechanisms can operate and hormones can regulate cellular functions; otherwise there would be overreaction, excessive feedback and desensitization in which receptors are down-regulated. An example of this is the continuous use of a GnRH agonist (leuprolide) to desensitize GnRH receptors in the anterior pituitary and decrease the release of LH and FSH (see Section 3.3). Hormones are inactivated in the target organs or by specialized organs, such as the liver. Removal or inactivation follows exponential decay kinetics (Fig. 1.21). The half-life of the hormones in the circulation is a measure of the longevity of hormone action. Many synthetic hormones and hormone analogues are designed to have a longer half-life and thus be effective for longer periods of time than natural hormones (see Section 2.3).

Peptide hormones are degraded by peptidases, such as the cathepsins in lysosomes, which split the peptide bonds in the molecule. Exopeptidases degrade protein from the carboxy-terminal end or the amino-terminal end. Endopeptidases, such as trypsin and chymotrypsin, degrade the protein at internal sites with some specificity. Trypsin hydrolyses peptide bonds where the carboxyl group is from lysine or arginine, while for chymotrypsin the carboxyl group in the peptide bond comes from phenylalanine, tryptophan or tyrosine. Deamination or reduction of disulfide bonds (e.g. insulin) can also inactivate proteins. This occurs in the kidney and liver and in target cell lysosomes.

Steroid hormones are degraded by a two-phase process in the liver and in the kidney. This process inactivates the steroids and makes them more water soluble for excretion. In phase one, enzymes such as cytochrome P450 add functional groups such as hydroxyl groups. These metabolites are then conjugated to glucuronic acid or sulfates by transferase enzymes (Fig. 1.22). These more water-soluble metabolites are excreted by the kidney in the urine or by the liver via the bile salts into the gastrointestinal tract. The enterohepatic circulation is important in recirculating some steroids back from the intestine into the circulation. In this process, steroid conjugates produced in the liver and excreted in bile are metabolized by gut microbes, which remove the conjugate to regenerate free steroid, which is reabsorbed into the blood (Fig. 1.23). The enterohepatic recirculation of steroids can potentially be manipulated by altering the gut microflora to alter metabolism or using binding agents to prevent reabsorption of the steroids.

However, there are other roles that have been suggested for steroid conjugates, rather than just degradation products for excretion. Sulfoconjugates are also thought to act as a storage form of steroid hormones, and 90% of dehydroepiandrosterone (DHEA) in the circulation is present as sulfoconjugate. This results in a longer half-life for the steroid, due to increased binding to plasma proteins, and also decreases the accumulation of the polar sulfoconjugate



Steroid sulfates

Steroid glucuronides

Fig. 1.22. Structure of steroid sulfates and glucuronides.

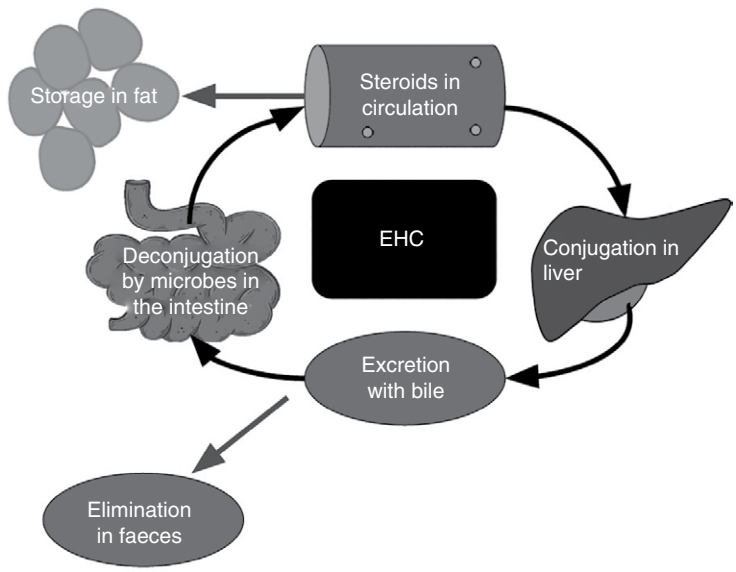


Fig. 1.23. Diagram of the enterohepatic circulation (EHC).

in fatty tissue. This may be important in the accumulation of odorous 16-androstene steroids in fatty tissue of pigs, leading to boar taint (see Section 3.3). The sulfatase enzyme regenerates free steroids at target tissues and increased sulfatase activity has been found in breast cancer tissue, which provides active oestrogen for the growing tumour from circulating oestrone sulfate.

1.3 Receptors and Hormone Action

Hormones interact with receptors located either on the cell surface or inside the cell to initiate their effects on the target tissue. Binding of hormones to cell-surface receptors results in immediate effects by activating intracellular enzyme systems to alter cell function. Hormones that cross the cell membrane act by binding to intracellular receptors. The hormone–receptor complex then interacts with DNA to cause slower effects by affecting expression of specific genes and *de novo* protein synthesis.

Extracellular receptors

Key concepts

- Hormones that bind to cell surface receptors activate a protein kinase, which phosphorylates specific intracellular proteins to alter their activity.
- Hormones that cross the cell membrane bind to intracellular receptors and the complex then interacts with DNA to affect expression of specific genes.
- Experimental evidence shows that cell surface receptors are large proteins and many are glycoproteins.
- Different types of heterotrimeric G proteins couple receptors to adenylate cyclase, phospholipase C or other effector molecules.
- Adenylate cyclase synthesizes the second messenger cyclic adenosine monophosphate (cAMP), which activates protein kinase A.
- Protein kinase A can also phosphorylate and activate CREB (cAMP-responsive element-binding protein), which binds to specific cAMP-responsive elements in regulatory regions to activate gene expression.
- Phospholipase C produces inositol phosphate and diacylglycerol to increase intracellular calcium and activate protein kinase C.
- Calmodulin binds calcium and is an allosteric regulator of protein kinase C and other enzymes.
- Catalytic receptors either have a kinase domain as part of the receptor structure or recruit a kinase to bind to the activated receptor.
- The tyrosine kinase receptor phosphorylates tyrosine residues in its kinase domain and then can phosphorylate other proteins or dock with SRC homology region 2 (SH2) domain adapter proteins.
- Cytokine receptors bind Janus kinase (JAK) tyrosine kinase, which phosphorylates the receptor and provides docking sites for signal transducer and activator of transcription (STAT) proteins, which activate the transcription of various genes.
- Receptor serine/threonine kinases phosphorylate SMAD proteins that translocate to the nucleus and modulate gene transcription.
- Mitogen-activated protein kinases (MAPK) integrate the cellular response to growth factors, cytokines and stresses.
- Hormone action is terminated by degrading the hormone, desensitization of receptors by phosphorylation, and endocytosis and degradation of the receptors.

Extracellular receptors are large transmembrane macromolecules located on the outer surface of the plasma membrane in target tissues. The transmembrane domain (TMD) of the receptor is a hydrophobic region anchored in the phospholipids of the membrane. Hormone binding to the hydrophilic extracellular domain (ECD) of the receptor stimulates signalling events at the intracellular domain (ICD) of the receptor inside the cell (Fig. 1.24).

For example, the insulin receptor has a molecular mass of 200–400 kDa and consists of two α-subunits of 130 kDa and two β-subunits of 90 kDa, linked by disulfide bonds. Usually, there are separate receptors for each hormone, and the function of the cell (i.e. the cell type) dictates whether a particular receptor will be present on a cell and the number of receptors present.

A number of experimental techniques can be used to demonstrate that a hormone receptor is located on the cell surface. A convenient model system for this work (see Section 2.1) would be a cell culture that responds to the hormones.

1. If treating the cells with antibodies against the receptor blocks hormone action, this would suggest that the antibodies are binding to the receptor on the cell surface to prevent hormone binding.

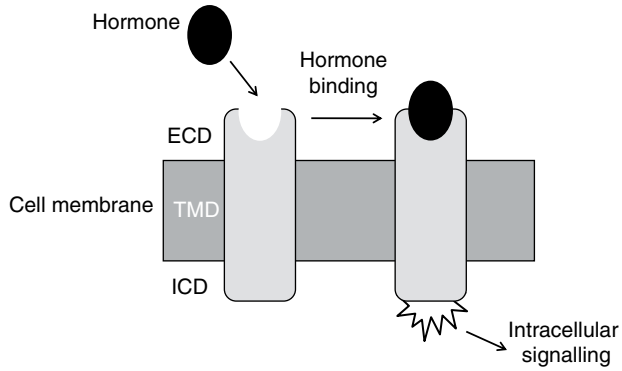


Fig. 1.24. General mechanism of action of cell surface receptors.

2. Limited proteolysis of intact cells would be expected to degrade the receptor on the cell surface and remove the hormone response.
3. If coupling the hormone to a large molecule that cannot enter the cell still results in a response to hormone treatment, this suggests that the hormone can still bind to the receptor on the cell surface.
4. Demonstrating that the receptor is present in a plasma membrane preparation produced by subcellular fractionation (100,000 *g* pellet).

Hydrophobic regions on the receptor protein interact with lipid in the membrane. The receptor can be solubilized with detergents and purified by affinity chromatography using the hormone bound to a column matrix. Receptors can be glycoproteins and contain carbohydrate residues. Experimental tools to demonstrate they are glycoproteins include the following.

1. Treat the receptor preparation with neuraminidase or β-galactosidase to remove the sugar residues. This inhibits binding of the hormone.
2. Concanavalin A (ConA) (a protein from jack bean that binds to D-glucosyl residues) can also be used to inhibit hormone binding. In addition, ConA can be used for affinity chromatography of glycoproteins (see 'Chemical assays' in Section 2.2).

The general mechanism of action for cell surface receptors (see Figs 1.5 and 1.24) involves hormone binding to the receptor on the outside of the cell, which then activates a protein kinase enzyme inside the cell that phosphorylates (adds a phosphate group to) specific intracellular proteins. Once phosphorylated, the activity of these proteins is altered and this ultimately produces a cellular response. The phosphate is removed from the protein by a protein phosphatase enzyme, which returns the protein to the resting state.

There are about 500 different protein kinases and 200 different protein phosphatases in the human genome. Some of these enzymes act on several protein substrates while others are specific for a single protein substrate. By phosphorylating and dephosphorylating substrate proteins, these enzymes modify the activity of up to 30% of all cellular proteins. Phosphorylation can either activate or inactivate enzymes and protein complexes, direct protein movement between subcellular compartments and initiate protein degradation. These changes are part of the overall signal transduction process, in which binding of hormones to receptors at the cell surface results in changes inside the cell.

There are several types of cell surface receptors. The G protein-coupled receptors (GPCRs) stimulate the synthesis of second messenger compounds, which activate intracellular kinases. The catalytic receptors include tyrosine kinase receptors, cytokine receptors and serine kinase receptors that either have a kinase domain as part of their structure or recruit a kinase to bind directly to the receptors after binding of the hormone.

G protein-coupled receptors

GPCR STRUCTURE AND FUNCTION G protein-coupled receptors (GPCRs) are by far the most common mechanism for transmembrane signalling, with more than 800 GPCRs in humans. They recognize a highly diverse set of ligands, including proteins, small molecules, hormones, drugs and photons of light, and are involved in nearly every aspect

of animal life, from early development and heart function to neuronal activity. They are very important to human medicine and GPCRs are targeted by about 35% of prescription drugs. Examples of GPCRs are adrenergic receptors, dopamine receptors, histamine receptors, the light receptor rhodopsin and the many odour and taste receptors (see Section 6.2). The effects of these receptors are also very diverse; epinephrine binding to the adrenergic receptor in heart and lung leads to activation, while binding to the adrenergic receptor in the gut leads to down-regulation of digestion. The Nobel Prize in Chemistry 2012 was awarded to Brian K. Kobilka and Robert J. Lefkowitz for studies of GPCRs

The transmembrane domain (TMD) of all of the GPCRs is a tertiary structure resembling a barrel, with the seven transmembrane α-helices linked by three extracellular loops (ECLs) and three intracellular loops (ICLs), which form a cavity within the plasma membrane (Fig. 1.25). This positions the N-terminus of the protein on the outside of the cell and the C-terminus inside. The N-terminus and ECLs are responsible for binding bulkier ligands (e.g. proteins or large peptides), while hydrophobic ligands are funnelled into a binding pocket formed by the upper half of the transmembrane domains.

Ligand binding causes conformational changes in the receptor protein which transfers the signal to the inside of the cell. Upon ligand binding, the seven transmembrane helices change conformation, with a twisting motion and outward movement of transmembrane helix 6. This opens a cytosolic cavity to form a wider intracellular surface and exposes residues of the intracellular helices, to form an active receptor conformation. This increases the affinity for binding of a GTP-binding protein

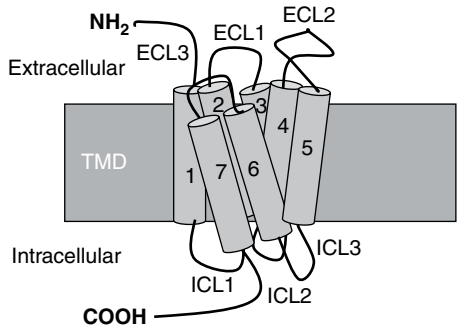


Fig. 1.25. Seven transmembrane structure of G protein-coupled receptors.

(G protein) to the receptor on the inside of the cell. The G protein is heterotrimeric and comprises three different α, β and γ subunits. Binding of the hormone to the receptor results in a change in conformation and an exchange of GDP with GTP on the Gα subunit (Fig. 1.26). The binding of GTP induces the Gα subunit to dissociate from the Gβγ subunits and activate a membrane protein (either adenylate cyclase or phospholipase C). In turn, the activated membrane protein stimulates the production of second messengers (cAMP or calcium), which activate a protein kinase and ultimately elicit the biological response in the cell. The activated receptor conformation lasts long enough to allow a bound ligand molecule to activate several G proteins, which amplifies the signal.

To return the system back to the inactive resting state (Fig. 1.27), the GTP bound to the Gα subunit is slowly converted to GDP by the GTPase activity of the Gα protein, and the α, β and γ subunits re-associate. The receptor is also phosphorylated by GPCR-regulating kinases (GRKs), at various serine/threonine residues of the third ICL and the C-terminal tail. This stimulates the binding of scaffolding proteins called β-arrestins, which prevent binding to G protein and promote endocytosis and internalization of the receptor into endosomes. The receptor can then be dephosphorylated and recycled to the cell surface to restart the cycle or be merged with a lysosome to be degraded.

GPCR signalling via G proteins can also be maintained after internalization and relocation into endosomes, depending on the subcellular localization of the internalized vesicle. Endosomal G protein signalling is linked with some physiological outcomes, such as Ca^{2+} metabolism for parathyroid hormone receptor (PTH1R) and chronic pain for neurokinin 1 receptor (NK1R). GPCRs can also signal from other intracellular compartments with distinct physiological outputs, including Golgi, ER, nucleus and mitochondria.

The GPCR is discrete from the G protein and enzyme system that it activates and they are located together within lipid microdomains or membrane rafts in the plasma membrane. These are regions of the outer leaflet of the plasma membrane which are enriched in cholesterol and other lipids that have greater order and less fluidity than other membrane regions. The co-localization of signalling components in membrane rafts may be due to interaction between the extracellular domains of the proteins, or interaction with the lipids or other membrane

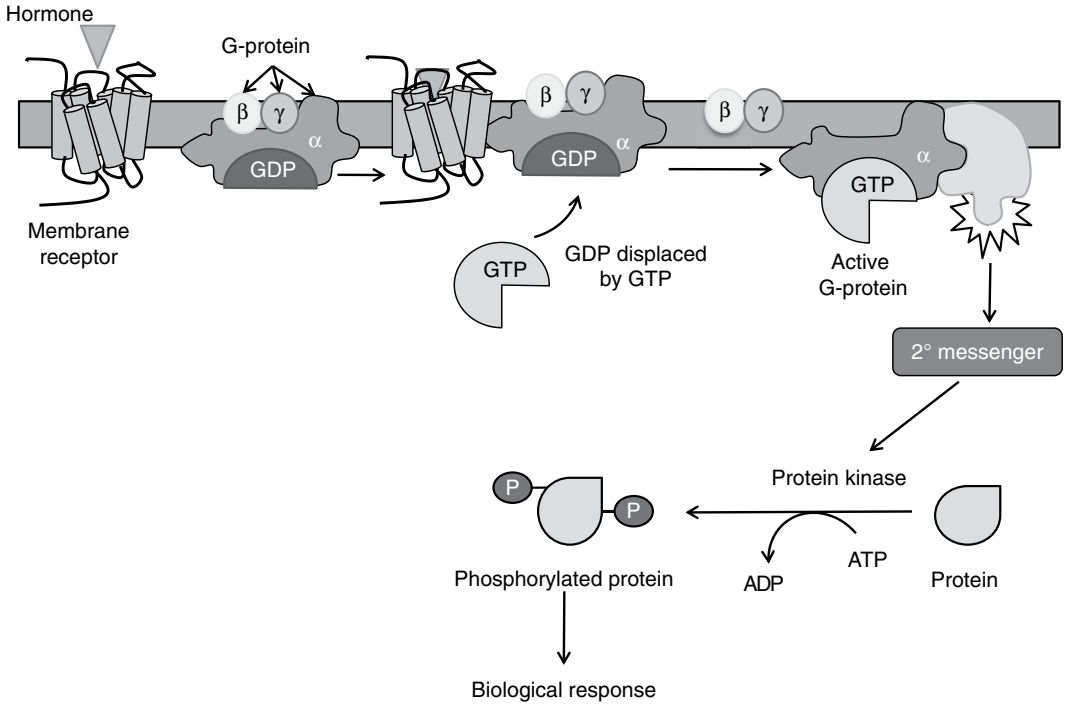


Fig. 1.26. General mechanism of activation of G protein-coupled receptors.

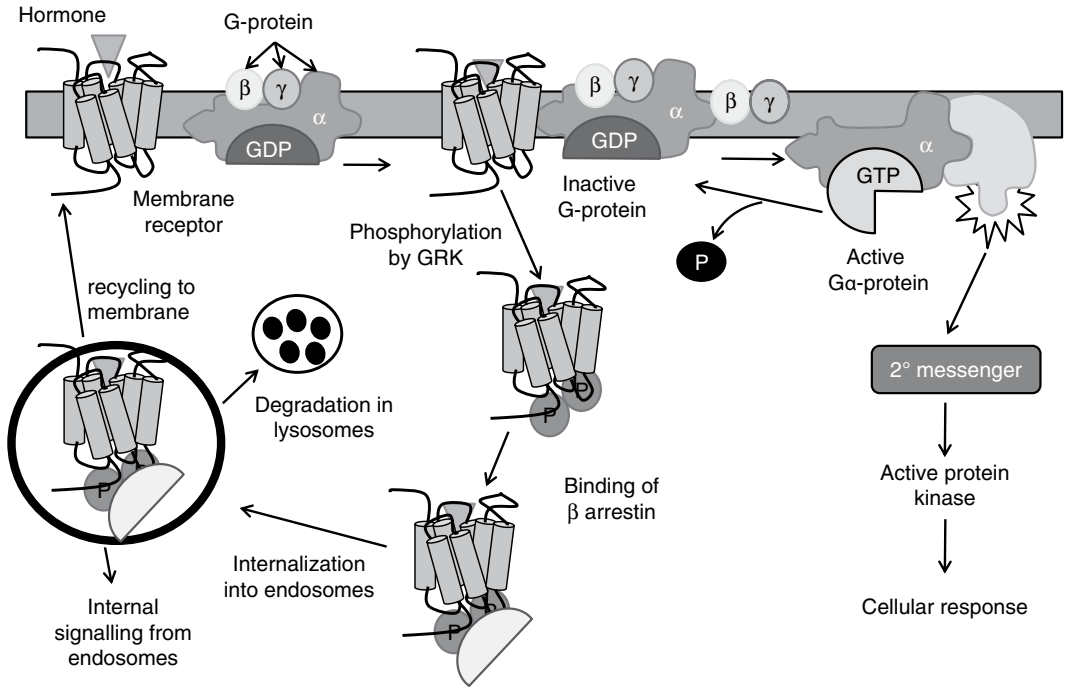


Fig. 1.27. General mechanism of deactivation of G protein-coupled receptors.

proteins in these regions. Co-localization of these proteins increases the efficiency of activation and amplification of the signalling pathways. Lateral movement of GPCRs within the plasma membrane is often restricted by the preferential localization to a specific lipid microenvironment. Although GPCRs are usually shown as monomers, they can form oligomers (i.e. homodimers and heterodimers) within lipid rafts. Membrane lipids may also enhance the recruitment of β-arrestins and subsequent internalization of the receptors. Once receptors are internalized to the endosomes, the specific endosomal lipids may stabilize the active state of the receptors and promote G protein coupling and activation.

Lipids can also modulate ligand binding and affect GPCR function. The same GPCR can also signal through different intracellular pathways depending on the identity of the bound ligand. This is due to the relative flexibility of the receptor in the membrane, which allows different ligands to stabilize different active or inactive forms. Phospholipids, in particular phosphoinositides, can directly bind GPCRs and potentially modulate their function. For further information see the reviews by Hanlon and Andrew (2015) and Sutkeviciute and Vilardaga (2020).

GPCR SIGNALLING PATHWAYS Endocrine signalling through GPCRs occurs by two main systems: (i) the adenylate cyclase–cAMP–protein kinase A (PKA) pathway; and (ii) the calcium-dependent phospholipase C–protein kinase C (PKC) pathway. In the first system, hormone binding to the receptor affects the activity of the enzyme adenylate cyclase, which synthesizes the second messenger cAMP, which activates protein kinase A. In the second system, binding of the hormone to the receptor activates phospholipase C, which splits phosphatidylinositol-4,5-bisphosphate (PIP2) in the cell membrane to produce inositol 1,4,5-phosphate (IP_3) and diacylglycerol (DAG). The inositol phosphate increases levels of intracellular calcium, which together with the diacylglycerol activates protein kinase C. Both protein kinase A and protein kinase C can phosphorylate and activate various intracellular proteins to alter cellular metabolism (refer to Fig. 1.36).

GPCRs utilize G proteins with different types of Gα subunits (Fig. 1.28): $G\alpha_s$, which stimulates adenylate cyclase to increase cAMP production; $G\alpha_i$, which inhibits adenylate cyclase to decrease cAMP production (e.g. β-adrenergic or somatostatin receptors); and $G\alpha_q$, which stimulates phospholipase C and increases intracellular calcium levels (see Fig. 1.34).

A number of experimental tools can be used to investigate G proteins (Table 1.2). Treatment with a non-hydrolysable form of GTP, GTPγS, permanently activates G protein and this can be used to demonstrate that a G protein is involved in a physiological response. Cholera toxin permanently activates $G\alpha_s$ and stimulates adenylate cyclase to increase production of cAMP, while pertussis toxin blocks $G\alpha_i$, preventing the inhibition of adenylate cyclase and subsequent reduction in cAMP. Thus, these reagents can be used to determine the involvement of which form of G protein is involved in a hormonal response.

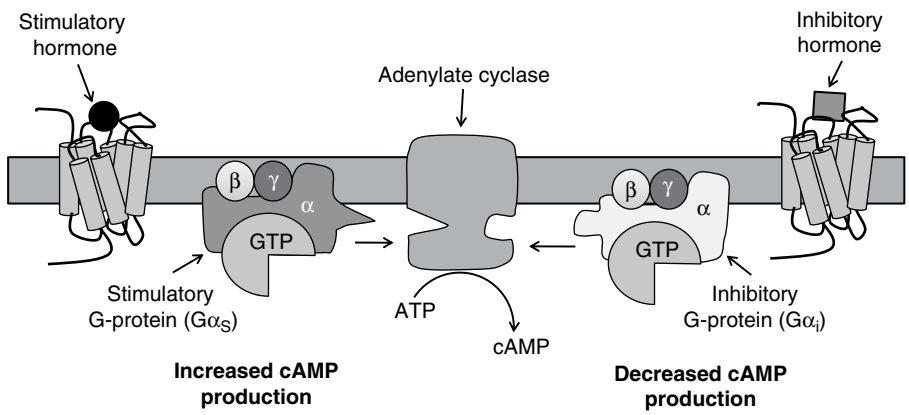


Fig. 1.28. Effects of stimulatory and inhibitory G proteins.

ADENYLATE CYCLASE–cAMP–PROTEIN KINASE A PATHWAY The enzyme adenylate cyclase catalyses the formation of cAMP from ATP. cAMP activates protein kinase A by binding to its regulatory subunit to release the active catalytic subunit. The active protein kinase A then phosphorylates intracellular proteins to alter their activity (Fig. 1.29). These proteins are inactivated by removal of the phosphate by the enzyme phosphoprotein phosphatase.

The formation of cAMP is an amplification step that increases the effective hormone concentration, since one adenylate cyclase enzyme catalyses the formation of many cAMP molecules. The enzyme phosphodiesterase degrades cAMP to AMP.

Activating protein kinase A and subsequent phosphorylation of intracellular proteins can cause immediate cellular responses, such as modification of the activity of metabolic pathways and regulation of ion flows. However, cAMP can also have effects on gene transcription (Fig. 1.30), since protein kinase A can translocate to the cell nucleus to phosphorylate the cAMP-responsive element-binding protein (CREB) or modify the structural proteins in chromatin. Phosphorylation at CREBSer111 and CREBSer121 inhibits transcription, while phosphorylation at CREBSer129 and CREBSer133 induces transcription. Hydrophobic leucine amino acids are located along the inner edge of the alpha helix of the

Table 1.2. Experimental tools to study GPCR systems.

GPCR system	Experimental tool	Response
Identification of G protein	Non-hydrolysable form of GTP, GTPγS	Permanently activates all G protein
	Cholera toxin	Permanently stimulates $G\alpha_s$
	Pertussis toxin	Blocks $G\alpha_i$
PKC/Ca/PLC pathway	Inhibit PLC with U73122	Blocks action of hormone
	Ca^{2+} selective ionophores or liposomes loaded with Ca^{2+}.	Increase intracellular Ca^{2+} to cause hormone response
	Chelating agent (EGTA), Ca^{2+} channel blockers.	Block effects of Ca^{2+} to reduce hormone response
	Inorganic Ca^{2+} antagonist	
	Phorbol esters (TPA)	Activate PKC to cause hormone response
PKA/ cAMP pathway	Forskolin (Fig. 1.32)	Activates adenyl cyclase to increase cAMP
	cAMP analogues (Fig. 1.31)	Cause hormone response
	Phosphodiesterase inhibitors (Fig. 1.31)	Maintain cAMP levels to potentiate the hormone response

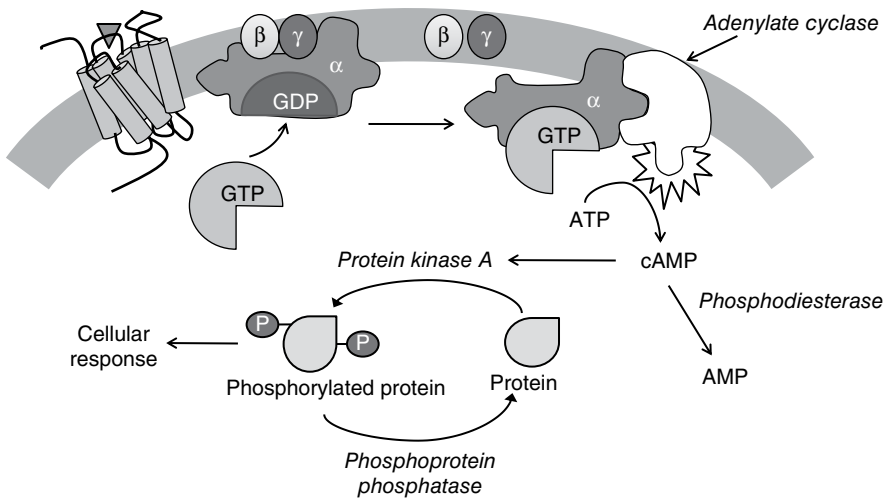


Fig. 1.29. Cyclic AMP second messenger system.

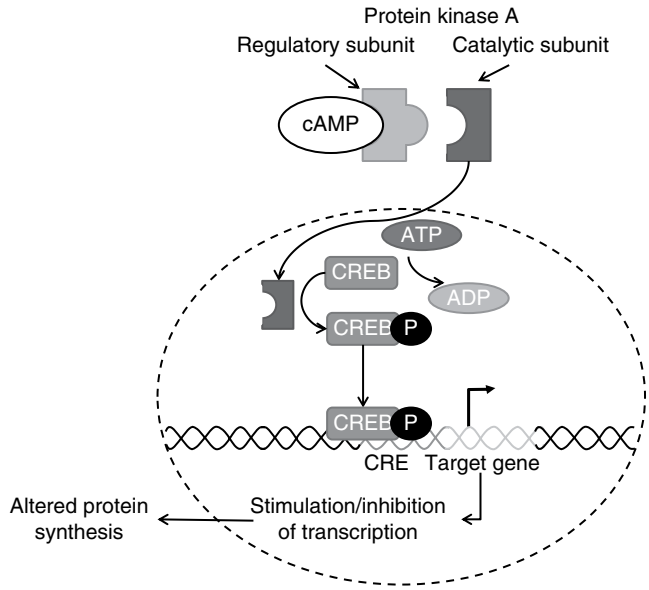


Fig. 1.30. Genomic actions of cAMP.

CREB protein and these bind to leucine residues of another CREB protein, forming a dimer. This chain of leucine residues forms the basic leucine zipper motif which is present in many gene regulatory proteins. Magnesium ion facilitates binding of the protein to DNA. Activated CREB binds to specific cAMP-responsive elements in the regulatory regions of certain genes to activate gene expression. When activated, the CREB protein recruits other transcriptional coactivators to bind to specific cAMP-responsive elements (CRE, which has the sequence 5′-TGACGTCA-3) in the regulatory regions of certain genes to regulate gene transcription, resulting in longer-lasting changes in cell function.

Endosomal cAMP signalling can prolong the cAMP signalling cascade when concentrations of circulating hormones are low, or facilitate the diffusion of cAMP into the nucleus to activate nuclear PKA and regulate CREB activity. For more information on CREB, see the review by Steven *et al.* (2020).

Several properties of cAMP make it suitable as a second messenger. It is derived from ATP but is chemically stable. ATP is ubiquitous and cAMP is formed from it in a single reaction. Since cAMP is not a metabolic precursor but an allosteric regulator, it can be controlled independently of metabolism. cAMP is a small and easily diffusible molecule and it has a number of functional groups, which allows specific binding to regulatory subunits of protein kinases.

The involvement of cAMP as the second messenger for a hormone can be determined experimentally (see Table 1.2). A convenient model system for this work (see Section 2.1) would be a cell culture that responds to the hormone.

- Treating the cells with physiological levels of hormone should increase cAMP levels in cells, and cAMP production should precede the physiological effect caused by hormone treatment.
- The hormone should stimulate adenylate cyclase activity in broken cells.
- Treatment of the cells with exogenous cAMP or its analogues, such as dibutyryl cAMP and 8-bromo cAMP (Fig. 1.31), should produce the hormone response (without treatment with the hormone).
- Treatment of the cells with phosphodiesterase inhibitors (Fig. 1.31), such as theophylline, caffeine or isobutylmethylxanthine (IBMX), will decrease cAMP clearance and thereby potentiate the hormone response.
- Treatment of the cells with the diterpene forskolin (Fig. 1.32), which binds directly with the catalytic subunit of adenylate cyclase to activate it permanently, will increase cAMP levels and produce the hormone response.

A number of different hormones act via the cAMP second messenger system (Table 1.3). The substrates

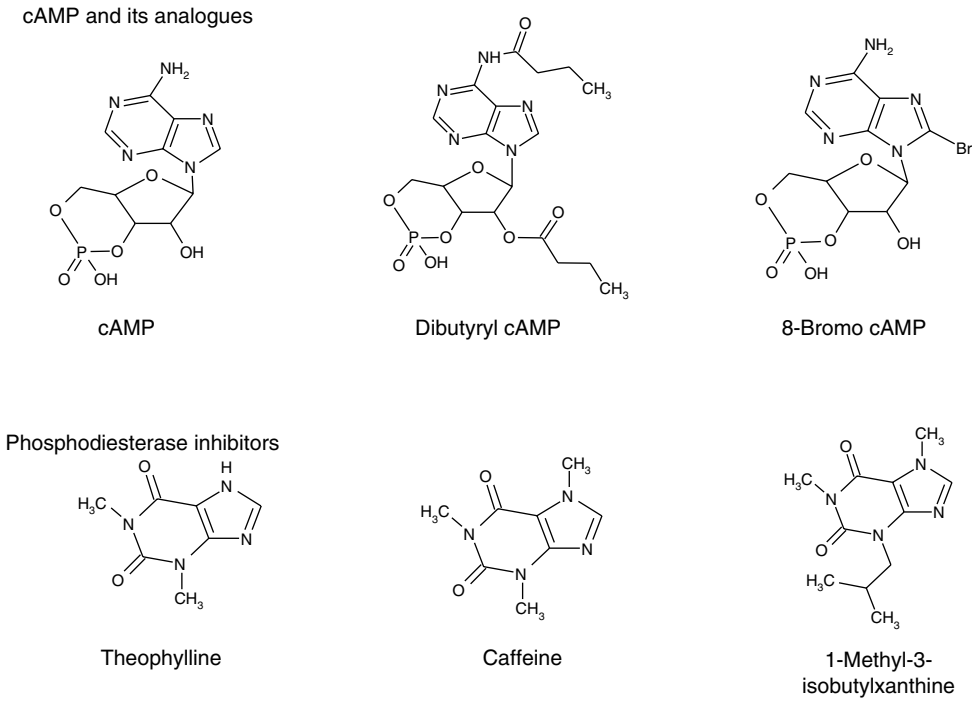


Fig. 1.31. cAMP and its analogues and phosphodiesterase inhibitors.

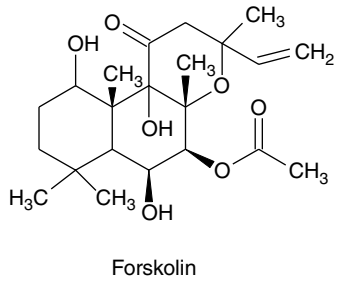


Fig. 1.32. Forskolin, an activator of adenylate cyclase.

for cAMP-dependent protein kinases include: triglyceride lipase, which is involved in the regulation of lipolysis; phosphorylase b kinase, involved in the regulation of glycogenolysis; cholesterol ester hydrolase, involved in the regulation of steroidogenesis; and fructose 1,6-diphosphatase, involved in the regulation of gluconeogenesis. These latter enzymes are all activated by phosphorylation. Enzymes that are inactivated by phosphorylation include: pyruvate kinase, involved in the regulation of glycolysis and gluconeogenesis; glycogen synthase, involved in the regulation of glycogen synthesis; and 3-hydroxy-3-methyl-glutaryl-CoA reductase, involved in the regulation of cholesterol biosynthesis.

A related system, the cyclic guanosine monophosphate (cGMP)-dependent protein kinase G system, may act in opposition to cAMP. For example, activation of the cAMP-dependent kinases results in smooth muscle relaxation, while activation of the cGMP-dependent kinases results in smooth muscle contraction. Levels of cGMP are normally 10–50 times lower than those of cAMP.

For interest

Rhodopsin has a central role in vision. It differs from many other 7TM receptors in that its ligand (retinal) is covalently bound, which allows rhodopsin to respond to the signal of the influx of light. Adsorption of a photon shifts the conformation of retinal from the *cis*- to the *trans*-isomer, which causes a conformational change of rhodopsin to the active conformation. Rhodopsin functions via a G protein (transducin), phosphodiesterase and cyclic GMP, resembling β-adrenergic receptor signalling via G protein, adenylate cyclase and cAMP.

CALCIUM-DEPENDENT PHOSPHOLIPASE C–PROTEIN KINASE C SYSTEM The primary intracellular effector in this pathway is calcium, which activates a calcium-dependent protein kinase C (PKC). Hormone binding activates G protein $G\alpha_q$ to activate phospholipase C, which catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol 1,4,5-phosphate (IP_3) and diacylglycerol (DAG) (Fig. 1.33).

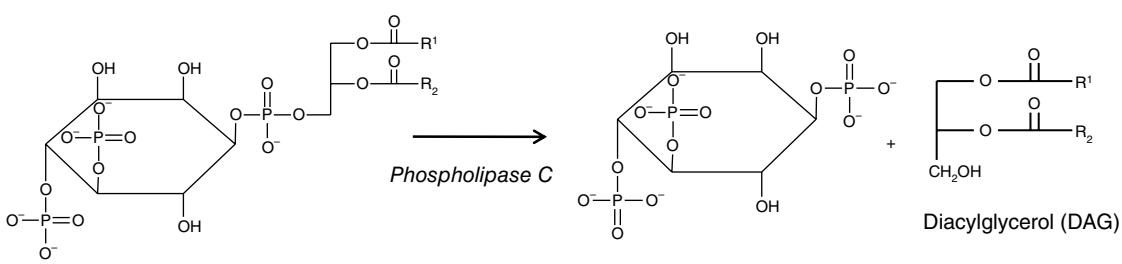
IP_3 and its metabolite, inositol 1,3,4,5-tetrakisphosphate (IP_4), are water soluble and increase intracellular Ca^{2+} by activating calcium channels at the endoplasmic reticulum (ER) to elicit Ca^{2+} release from the ER and at the cell surface. DAG is lipid soluble and diffuses along the plasma membrane, where it activates membrane-localized forms of protein kinase C (PKC) by increasing its affinity for Ca^{2+}. The activated PKC then phosphorylates cellular proteins to regulate their activity (Fig. 1.34).

Calcium also binds to calmodulin (CaM) to form an active complex. CaM is a heat-stable globular protein of molecular weight 16.7 kDa and is a calcium-dependent regulatory protein found in all eukaryotic cells. It binds four Ca^{2+} ions per molecule, to form an active complex which acts as an allosteric regulator of PKC and other enzymes. It also controls the activity of cellular filamentous organelles (actin and myosin) responsible for cell motility, exoplasmic flow (hormone secretion) and chromosome movement. CaM is also a regulatory subunit of adenylyl cyclase and phosphodiesterase in the cAMP signalling pathway.

Hormones that signal through the phospholipase C–PKC system include angiotensin II, catecholamines (epinephrine and norepinephrine), growth hormone-releasing hormone (GHRH), vasopressin, gonadotropin-releasing hormone (GnRH) and thyroid-releasing hormone.

Table 1.3. Some hormones that act via the adenylate cyclase–cAMP–protein kinase A pathway.

Hormone	Target tissue	Response
Glucagon	Liver	Glycogenolysis
	Liver	Gluconeogenesis
	Pancreas	Insulin secretion
Vasopressin	Kidney medulla	Water reabsorption
Thyrotrophin (TSH)	Thyroid	Thyroglobulin hydrolysis
Adrenocorticotrophic hormone (ACTH)	Adrenal cortex	Steroidogenesis
Luteinizing hormone (LH)	Corpus luteum	Steroidogenesis
Follicle-stimulating hormone (FSH)	Ovary	Steroidogenesis
Chorionic gonadotrophin	Ovary	Steroidogenesis
Parathyroid hormone	Renal cortex	Phosphaturia
	Bone	Calcium resorption ↑
Calcitonin	Bone	Calcium resorption ↓
Thyrotrophin-releasing hormone (TRH)	Anterior pituitary	TSH release
Secretin	Exocrine pancreas	Bicarbonate secretion
Epinephrine and norepinephrine	Muscle, adipose	Glucose metabolism



Phosphatidylinositol 4,5-bisphosphate (PIP2)

1,4,5-Inositol triphosphate (IP3)

Fig. 1.33. Action of phospholipase C.

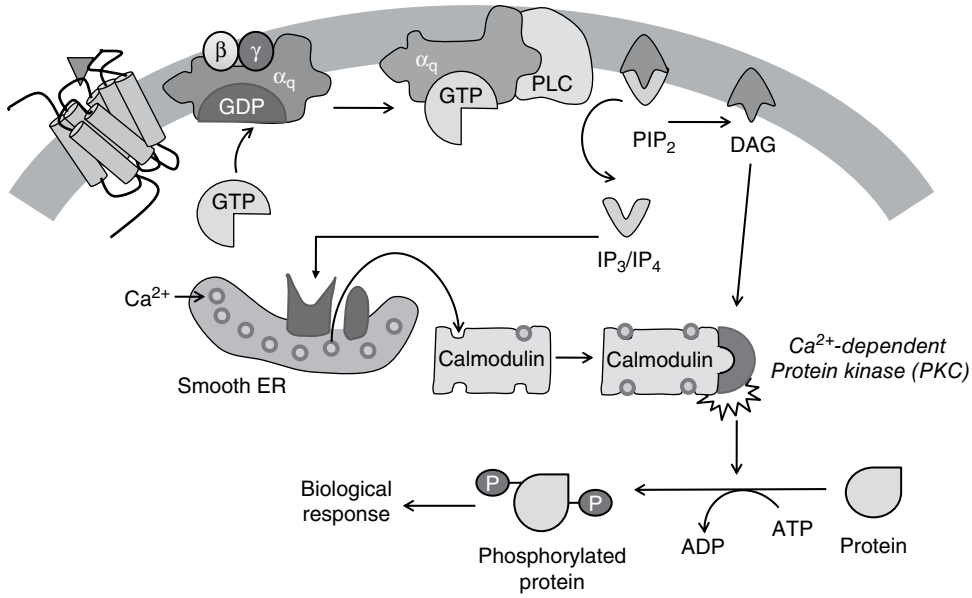


Fig. 1.34. Calcium-dependent protein kinase C second messenger system.

There are a number of experimental tools that can be used to determine the involvement of the calcium-dependent phospholipase C–PKC system in a hormone response (see Table 1.2). Again, a convenient model system for this work (see Section 2.1) would be a cell culture that responds to the hormone.

1. Increasing intracellular Ca^{2+} levels by treating the cells with Ca^{2+}-selective ionophores (A23187) or liposomes loaded with Ca^{2+} would activate PKC and cause the hormone effect.
2. Decreasing intracellular Ca^{2+} levels by treating cells with chelating agents such as ethylene glycol tetra-acetic acid (EGTA), using Ca^{2+} channel blockers, or inorganic Ca^{2+} antagonist (La^{3+}) would decrease PKC activity in the cells and decrease the response to hormone treatment.
3. Treating the cells with phorbol esters (TPA) which resemble diacylglycerol (Fig. 1.35; see structure inside dashed lines) will activate PKC in the cells and cause the hormone response.
4. Treating cells with U73122 will inhibit phospholipase C (PLC) and prevent the hormone response; the inactive analogue U73343 is used as a negative control.

INTERACTION OF cAMP AND CA^{2+} PATHWAYS There is a considerable amount of 'cross-talk' between the

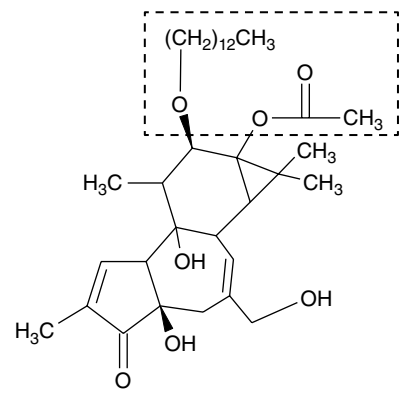


Fig. 1.35. Structure of tetradecanoylphorbol acetate (TPA) or phorbol 12-myristate 13 acetate (PMA). The outlined area has a structure similar to that of diacylglycerol (DAG).

different secondary messenger systems. Ca^{2+} binds to calmodulin and this complex can bind to phosphodiesterase to activate it and decrease cAMP levels. Protein kinase A (PKA), which is activated by cAMP, can phosphorylate some Ca^{2+} channels and pumps and alter their activity to affect intracellular calcium levels. Protein kinase C (PKC) can be phosphorylated by protein kinase A to change its activity. Protein kinase C and protein kinase A can phosphorylate different sites on the same protein,

so that its activity is regulated by both cAMP and Ca^{2+}. Both the cAMP and Ca^{2+} pathways result in activating protein kinases that phosphorylate target proteins to cause physiological effects (Fig. 1.36).

Catalytic receptors

The cell surface catalytic receptors do not use a second messenger system to activate a separate intracellular protein kinase but either have a kinase domain as part of the receptor structure or recruit a kinase to bind to the activated receptor. They include: the tyrosine kinase receptors, which activate their intracellular kinase domain by autophosphorylation of tyrosine residues; the cytokine receptors, which recruit the JAK tyrosine kinase; and the serine/threonine kinase receptors, which have their own intrinsic serine/threonine kinase activity. These receptors regulate long-term functions of cells by altering the expression of various genes, leading to changes in cell division, differentiation or cell turnover. Mutations in these receptors can contribute to the initiation and growth of certain cancers.

TYROSINE KINASE RECEPTORS The tyrosine kinase receptors do not use a second messenger system to activate a separate protein kinase but have a kinase domain as part of the receptor structure. The activated receptor phosphorylates tyrosine residues in its kinase domain and then can phosphorylate other proteins and is thus called a receptor tyrosine kinase (RTK). The receptor consists of a transmembrane domain, an extracellular domain for hormone recognition and a cytoplasmic domain that transmits the regulatory signals and contains adenosine triphosphate (ATP) binding sites. The cytoplasmic domain has a C-terminal tail with autophosphorylation sites. The phosphorylated receptor acts as a kinase enzyme and phosphorylates substrates. These phosphorylated substrates transmit several regulatory signals into the cell.

There are several classes of tyrosine kinase receptors (Fig. 1.37). The Class I receptor, the epidermal growth factor (EGF, ErbB) receptor, is a monomeric transmembrane protein with intracellular and extracellular domains on the same molecule. The extracellular domain contains two cysteine-rich repeat regions. Insufficient ErbB signalling is associated with the development of neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease in humans and defects in organs, including the lungs, skin, heart and brain. Excessive ErbB signalling is associated with the development and malignancy of a wide variety of types of solid tumours.

The Class II receptor, which is activated by insulin, IGF-1 and IGF-II, is a heterotetrameric receptor in which the two α-subunits and the two β-subunits are linked by disulfide bonds. It plays a key role in the regulation of glucose homeostasis. Autophosphorylation of various tyrosine residues within the intracellular β-chain facilitates the recruitment of specific adapter proteins such as the

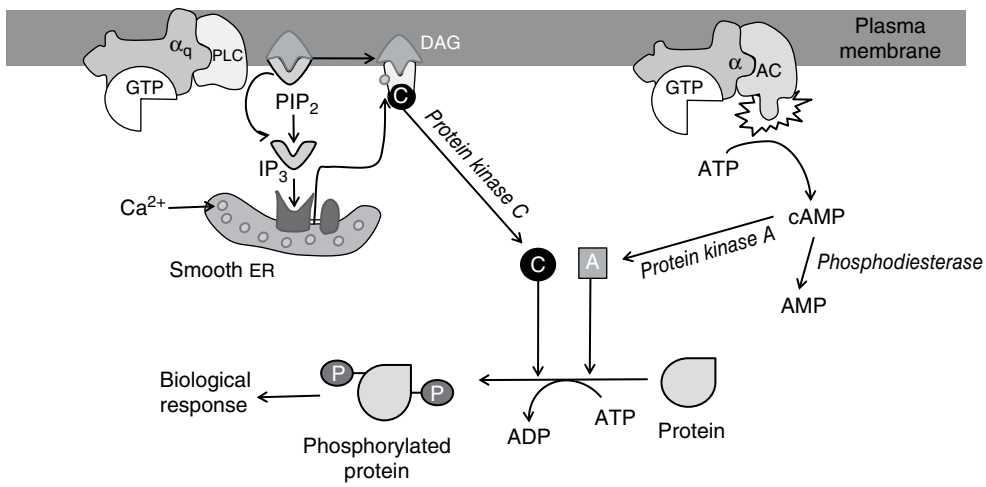


Fig. 1.36. Comparison of PKA and PKC pathways.

insulin receptor substrate (IRS), which acts as a secondary messenger within the cell to stimulate the transcription of insulin-regulated genes.

The Class III receptors, which bind the platelet-derived growth factor (PDGF) family and nerve growth factor (NGF), are monomeric proteins of two forms: alpha and beta. These receptors are important factors regulating cell proliferation, cellular differentiation, cell growth, development and many diseases, including cancer.

Hormone binding causes dimerization of the monomeric receptors (Fig. 1.38). The kinase domains in the monomers are then phosphorylated and activated by their partner. The kinases can then phosphorylate and activate other proteins. The receptors also interact with proteins containing Src

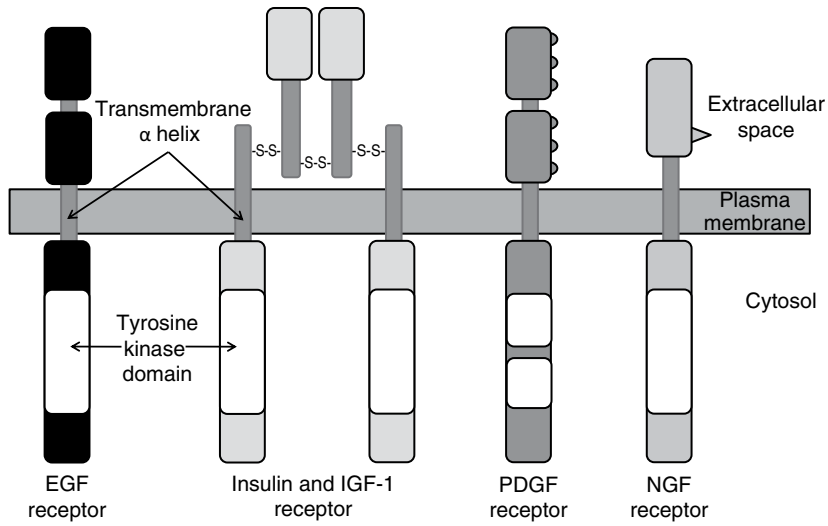


Fig. 1.37. Types of tyrosine kinase receptors.

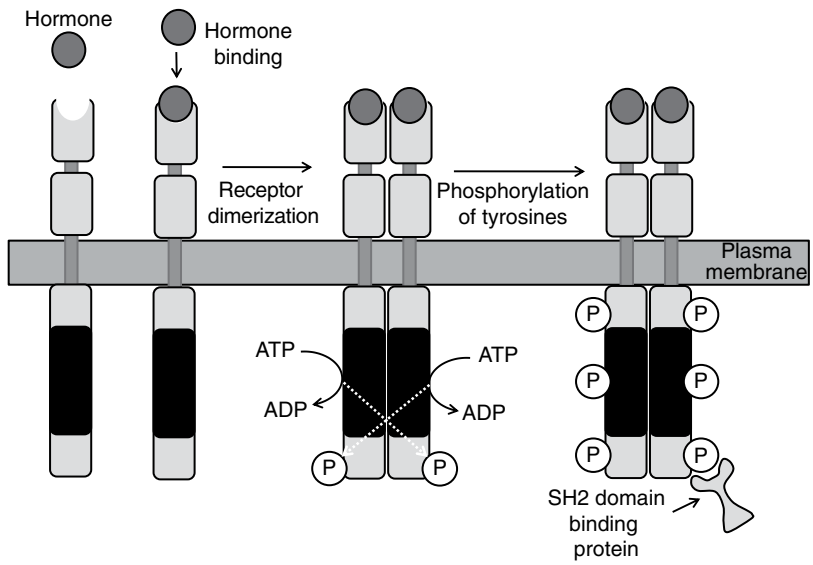


Fig. 1.38. Mechanism of action of tyrosine kinase receptors.

homology 2 (SH2) domains that are 100 amino-acid residues long and that bind to phosphotyrosines. Binding to phosphotyrosine occurs at an 'FLVR' amino acid motif, with the arginine the most critical residue for binding. The specificity of binding to the receptor is determined by the amino acid that is three residues C-terminal to the phosphotyrosine binding site (termed the +3 position). The SH2 domain protein is found in transcription factors and can be attached to a different enzymatic domain or can be a linker molecule that binds to other enzyme molecules that could not normally bind to the receptor. In this way, linker molecules can bind a number of different specific molecules to bring them together as part of an 'interactome' to produce the desired biological effect. For example, the appropriate kinase and phosphatase can be held in position so that a protein (receptor, ion channel, etc.) is activated by the kinase in the presence of the appropriate signalling molecule and is deactivated by the phosphatase when the signalling molecule is absent. SH2 adapter molecules also activate a monomeric G protein Ras, which then activates the MAPK kinase cascade (see Fig. 1.41 below). For more information on the role of SH2 domain proteins, see the review by Chehayeb and Boggon (2020).

For interest

A number of oncogenes code for components of endocrine signalling systems. The drug Imatinib (Gleevec® or Glivic® from Novartis), used in cancer treatment, is a competitive inhibitor of the bcr-abl tyrosine kinase, which is required for growth of tumour cells in chronic myelogenous leukaemia.

CYTOKINE RECEPTORS There are two classes of receptors for cytokines. Class I cytokine receptors include those for erythropoietin (EPO), prolactin (PRL), growth hormone (GH), thrombopoietin (TPO), leptin (LEP), granulocyte-macrophage colony-stimulating factor (GM-CSF), leukaemia inhibitory factor (LIF) and interleukin-3 (IL-3), IL-5, IL-6 and IL-7. They share a common amino acid motif (WSXWS) in the extracellular portion adjacent to the cell membrane. The Class II cytokine receptor family includes the interferon receptors, and receptors for interleukin-10, IL-19, IL-20, IL-22, IL-24, IL-26 and IL-29.

These receptors do not have intrinsic kinase activity but have a non-receptor Janus kinase (JAK) tyrosine kinase associated with a proline-rich region in each intracellular domain adjacent to the cell membrane (Fig. 1.39). The receptor exists as an inactive dimer when it is not bound to hormone. The binding of hormone causes a conformational change in the receptor and rotation of the intracellular domains to align JAK tyrosine kinase molecules bound to each receptor monomer. The JAK tyrosine kinases then phosphorylate the receptor on specific tyrosine residues, which act as docking sites for phosphorylation and activation of intracellular signalling molecules – STATs (signal transducers and activators of transcription). The activated STATs dissociate from the receptor and form dimers before translocating to the cell nucleus, where they regulate transcription of selected genes.

RECEPTOR SERINE/THREONINE KINASE Receptor serine/threonine kinases (RSTK) include receptors for transforming growth factor-β (TGFβ), bone morphogenic protein, activin and inhibin. These peptide growth factors are involved in the control of cell proliferation and differentiation.

There are two types of receptors and the binding of hormone results in formation of a heterodimer of receptor types I and II. After binding, the kinase activity of receptor II phosphorylates a glycine-serine (GS) regulatory region in the intracellular domain of receptor I (Fig. 1.40). The activated receptor then phosphorylates Smad (suppressor of MAD (mothers against decapapentaplegia)) proteins, which will dimerize, translocate to the nucleus and modulate gene transcription. For more information, see the reviews by Massague (2012) and Botello-Smith *et al.* (2017).

MAPK SYSTEM The mitogen-activated protein kinases (MAPKs, also known as extracellular signal regulated kinases (ERKs)) are serine/threonine protein kinases that regulate a wide variety of cellular processes. They phosphorylate transcription factors and other proteins that regulate cell proliferation, survival, motility, metabolism, transcription and translation. The MAPK/ERK pathway has been implicated in the development of many cancers.

The process begins when an extracellular mitogen or growth factor binds to a tyrosine kinase membrane receptor, such as the epidermal growth factor receptor. The monomeric G protein Ras is then activated by adapter molecules that bind to

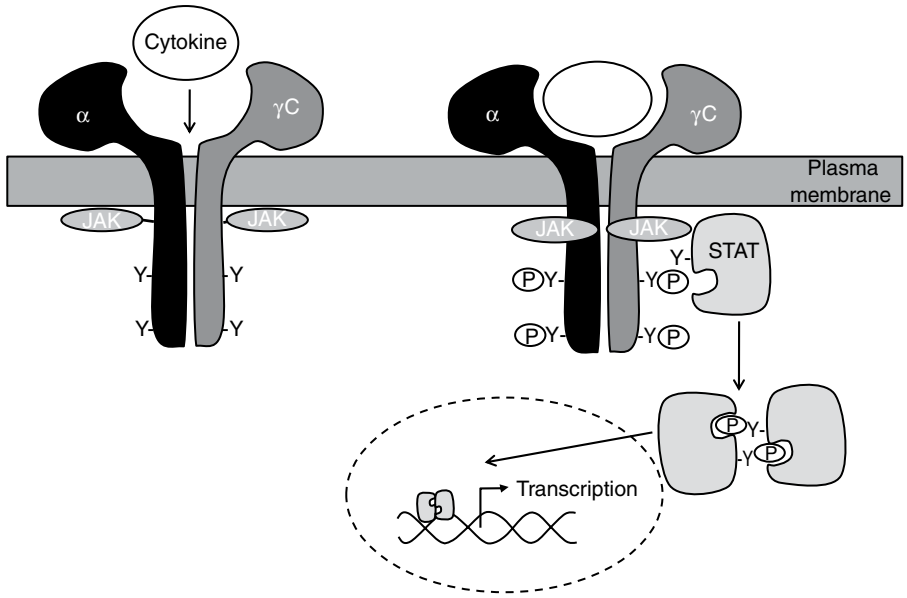


Fig. 1.39. Mechanism of action of cytokine receptors.

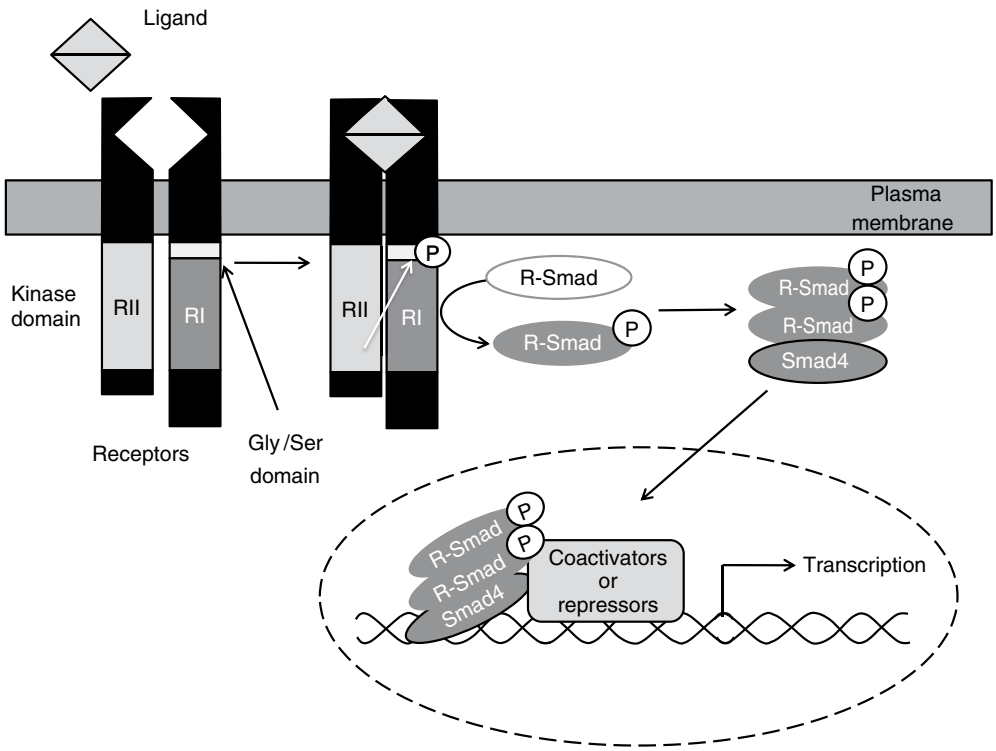


Fig. 1.40. Mechanism of action of receptor serine/threonine kinases.

the activated tyrosine kinase receptors (see discussion above). Ras can now activate a cascade of kinases: MAP3K, which activates MAP2K, which activates MAPK. The activated MAPK can now activate a transcription factor, such as Myc (Fig 1.41), and alter the transcription of genes that are important for the cell cycle. MAPK activation can also alter the translation of mRNA to proteins. The cascade can also be activated by protein kinase C and other G proteins. MAPKs thus are involved in the cellular response to growth factors, cytokines and stresses, and the MAPK system can integrate a variety of extracellular signals. For more information, see the review by Braicu *et al*. (2019).

Termination of hormone action

The termination of hormone action and return of the system to the basal state is necessary to prevent overstimulation and eventual down-regulation of the hormone system. Rapid desensitization of GPCR receptors can occur by phosphorylation from specific GRK protein kinases, for example the β-adrenergic receptor kinases and rhodopsin kinase. Phosphorylation also increases the affinity of the receptor for regulatory proteins, β-arrestins, leading to further down-regulation. Binding of β-arrestin to the cytosolic domain of the receptor results in blockage of the G protein-binding site and desensitization of the receptor (see Fig. 1.27). After hormones interact with receptors, they can cluster together in clathrin-coated pits and this triggers vesicularization of membrane and endocytosis to form endosomes (Fig. 1.42). The hormone can dissociate from the receptor and the available hormone is degraded. The receptors may then be degraded by lysosomal enzymes or the receptor can be recycled to the cell surface. The hormone at the cell surface can be degraded by serum enzymes. Finally, the phosphorylated proteins are dephosphorylated by specific phosphoprotein phosphatase enzymes.

Intracellular receptors

Key concepts

- Nuclear receptors for steroid hormones act as transcription factors. They have common structural domains for hormone binding, binding of the hormone receptor complex to DNA and gene activation to stimulate gene transcription.
- The DNA-binding domain of the receptor contains 'zinc fingers', an amino acid motif present in transcription factors that binds to specific hormone response elements (HREs) in the target gene.
- HREs consist of pairs of hexamers arranged as inverted palindromes or direct repeats separated by a defined number of intervening bases.

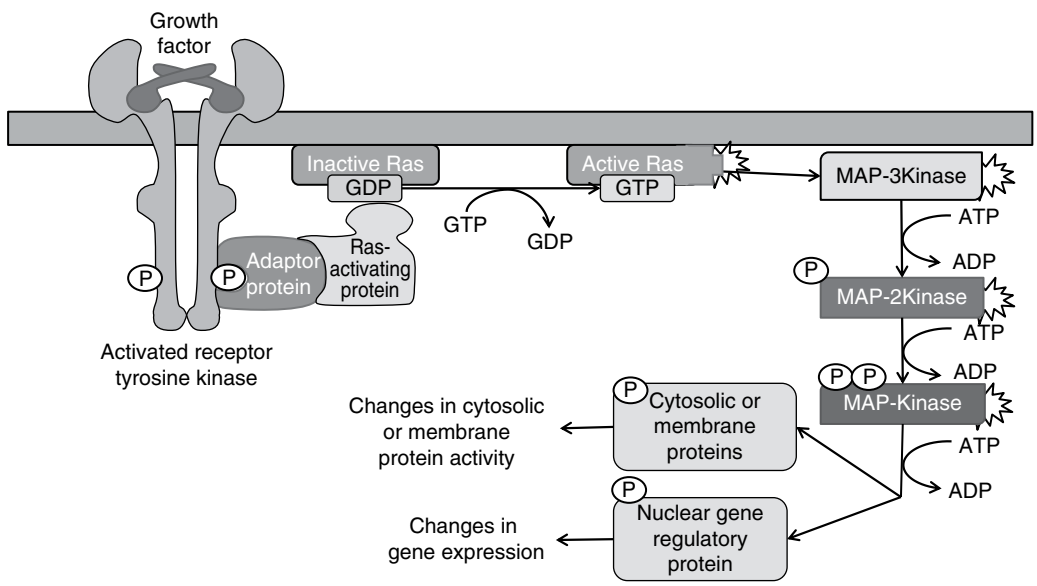


Fig. 1.41. The MAPK kinase cascade.

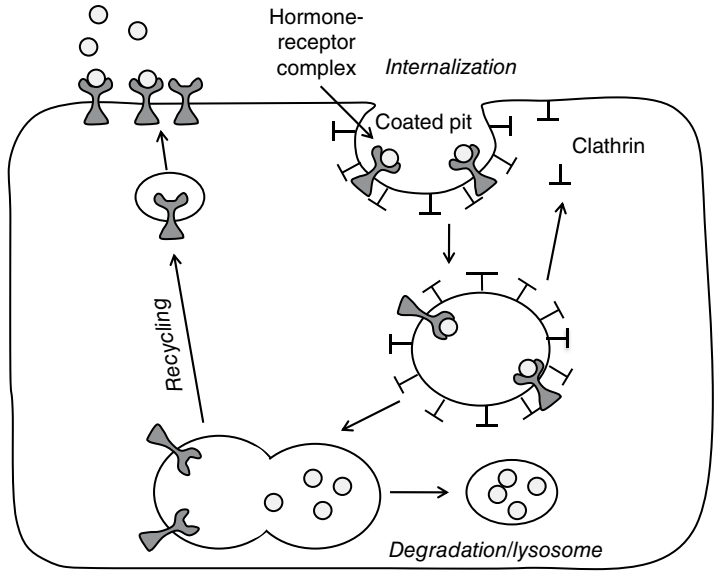


Fig. 1.42. Internalization, degradation and recycling of cell surface receptors.

- Both histones and high-mobility group (HMG) proteins affect the structure of chromatin.
- When the hormone–receptor complex binds to DNA, it interacts with other transcription factors that activate RNA polymerase and gene transcription.
- DNA regulatory sequences can be identified using gene reporter assays. DNA-binding proteins and their binding sites on DNA can be identified by DNase footprinting, EMSA and ChIPseq assays.
- Steroid hormones can also have non-genomic effects and integrate with the effects of peptide hormones.

Steroid and thyroid hormones operate via intracellular or nuclear receptors (NRs). In 1966, Toft and Gorski identified oestrogen receptors, using radiolabelled hormone, and showed that the receptor was only present in target cells. The first member of this NR family was cloned in 1985. In mammals, 48 NRs have been identified which bind a wide variety of small lipophilic ligands, including endocrine steroids, fat-soluble vitamins A and D, thyroid hormone, fatty acids, oxysterols, bile acids and a wide range of xenobiotic compounds from the diet (see below). They act as transcription factors that regulate the expression of genes involved in: reproduction, development, immune response, growth and circadian rhythm; nutrient uptake, metabolism and excretion; and drug and xenobiotic metabolism. NRs also integrate the responses among these different systems; for example, the NRs constitutive androstane receptor (CAR) and pregnane X receptor (PXR) that respond to xenochemicals not only regulate the metabolism of these compounds but also interact with metabolic pathways for nutrients and affect energy homeostasis. They can thus modulate the effects of xenobiotic endocrine disruptors (see Section 6.4)

NRs all share highly conserved functional domains involved in ligand binding, DNA binding and transcription activation (see section below for more details). Starting in the late 1980s, researchers used this information to search for additional NRs without prior knowledge of a naturally occurring ligand; these new members were therefore referred to as 'orphan receptors'. These orphan NRs were identified by screening cDNA libraries using probes designed from conserved NR functional domains, using degenerate primers for target amplification by PCR, as well as automated searches of DNA databases. While endogenous and synthetic ligands have since been discovered for many of the orphan NRs, the physiological role of some of these endogenous ligands has not been established and some orphan NRs are likely ligand-independent transcription factors. These orphan NRs regulate dozens of diverse physiological processes, from embryonic

stem cell self-renewal and differentiation to inflammation, circadian rhythm and metabolism.

The newest generation of NR ligands are termed 'selective nuclear receptor modulators', which are designed against a single NR to act as antagonists or agonists that affect a subset of signalling pathways in a tissue- or gene-selective manner. The goal is to separate the beneficial outcomes of treatment from the less desirable side effects and this has led to the development of drugs that target a range of disorders (see Section 3.2). Receptor modulators have been developed for targeting ER, AR, PR, VDR, TR, GR, PPARγ and LXR (see following section). For more information, see the review by Burris *et al*. (2013).

Nuclear receptor superfamily classification

The NRs are divided into seven subgroups: NR0 to NR6.

Subgroup 0: This group includes the NR02Bs, dosage-sensitive sex-reversal–adrenal hypoplasia congenital critical region on the X chromosome, Gene 1 (*DAX1*), and small heterodimer partner (SHP). These two proteins lack a classic DNA-binding domain (DBD) and contain only a ligand-binding domain (LBD) that contains motifs that are commonly seen in NR coactivators. These motifs interact with the activation function-2 region (AF-2) (see Fig. 1.45) of other NRs, preventing coactivator recruitment while recruiting co-repressor complexes and acting as transcriptional repressors.

Subgroup 1: This large family includes thyroid hormone receptors (TRs), retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs), reverse-Erb receptors (REV-ERBs), retinoic acid-related receptors (RORs), farnesoid X receptors (FXRs), liver X receptors (LXRs) and vitamin D receptors (VDRs). These receptors are regulated by a variety of lipophilic signalling molecules, including thyroid hormone, fatty acids, bile acids and sterols.

Peroxisome proliferator-activated receptors (PPARs, NR1C group) act as 'sensors' for a wide range of fatty acid molecules to regulate carbohydrate and lipid metabolism. Activation of PPARα and PPARβ/δ increases fatty acid oxidation in the heart, liver and muscle, while PPARγ promotes lipid storage in adipose tissue.

The REV-ERB, or Rev-Erb, NR1D group was discovered as a coding sequence on the reverse strand of the c-erbα gene, which encodes thyroid hormone receptor. They lack a critical alpha-helix in the AF2 domain required for coactivator interaction, and thus act as dedicated transcriptional repressors. Rev-Erb expression is tightly controlled by the core circadian clock and they act as integrators of circadian rhythm and metabolic pathways (see Section 1.4 for details of the circadian clock).

The RAR-related orphan NR1F group act as constitutive activators of transcription. They bind as monomers to the same sequence bound by Rev-Erbs. Retinoids and retinoic acid antagonize the activity of this NR.

The NR1H group, which includes liver X receptor (LXR) and farnesol X receptor (FXR), have metabolic roles that minimize the accumulation of cholesterol. LXR binds a number of cholesterol sterols to prevent cholesterol overload in a number of tissues. FXR is a central regulator of bile acid homeostasis and activation of FXR initiates a negative feedback loop that limits bile acid synthesis and promotes their transit from hepatocytes to the enterohepatic circulation.

The NR1I receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) play a key role in the metabolism of xenobiotics. PXR binds a diverse array of xenobiotics and dietary compounds, while CAR is constitutively active but also binds a number of phenobarbital-like compounds. Both receptors induce phase I and II enzymes in the liver and intestine that are important in xenobiotic and endobiotic metabolism.

Subgroup 2: This subfamily contains orphan receptors such as the retinoid X receptors (RXRs), chicken ovalbumin upstream promoter transcription factors (COUP-TFs) and hepatocyte nuclear factor 4 (HNF4). These orphans bind fatty acids, but it is not clear how these fatty acids regulate the activity of the receptors. The hepatocyte nuclear factors (HNF4, NR2A group) are required for the maturation, maintenance and differentiated functions of hepatocytes. Disruption of the receptor results in hepatomegaly, fatty liver, reduced serum cholesterol and triglycerides, increased serum bile acids and premature death.

RXRs (NR2B group) are retinoic acid-responsive factors that have some homology with retinoic acid receptors (RARs). RXRs bind 9-*cis*-retinoic acid with high affinity and dimerize with and strengthen the DNA-binding and transcriptional activity of other NRs, including TR, RARs, VDR, PPARs, LXRs, FXR, PXR, CAR, NGFIB and NURR1. Because of this wide array of binding partners, RXRs can affect the biological activity of many NRs.

Testicular NR2C (Tr2/4) is expressed in embryonic and adult tissues, suggesting multiple physiological functions. There is some evidence that it can bind and be activated by polyunsaturated fatty acids.

The photoreceptor NR (PNR, NR2E group) regulates the development of the rods and cones of the retina and plays a critical role in the development of the limbic system, where deletion leads to extreme aggression.

The NR2F chicken ovalbumin upstream promoter TF1 (COUP-TFI) and ErbA-related protein 2 (EAR2) act as transcriptional repressors. They are true orphan NRs that can compete with and antagonize the action of other NRs. They have essential functions in neural development and organogenesis.

Subgroup 3: This group comprises the steroid receptors (SRs), which are key regulators of a host of metabolic, reproductive and developmental processes. The SR family includes the androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and two closely related oestrogen receptors (ERα and ERβ). Cholesterol-derived hormones, like cortisol and oestrogen, regulate SRs through direct binding.

NR3B oestrogen-related receptors (ERRs) were the first orphan NRs to be discovered by screening libraries with the sequence of the DBD of oestrogen receptor. They are constitutively active and do not bind ligand but are affected by inverse agonists that induce a conformational change favouring interaction with co-repressors. They regulate genes that modulate cellular energy metabolism by directing mitochondrial biogenesis and function.

Subgroup 4: The NR4A group contains the orphan nuclear receptors nerve growth factor 1B (NGF1-B), nurr-related factor-1 (NURR1) and neuron-derived orphan receptor-1 (NOR-1). These proteins function as developmental modulators or immediate early genes whose expression is rapidly induced by a variety of stimuli to control cellular proliferation, function, or death and are required for neuronal development and maintenance.

Subgroup 5: The NR5A group of receptors, steroidogenic factor-1 (SF-1) and liver receptor homologue-1 (LRH-1), are transcriptional activators that have large, hydrophobic ligand-binding pockets that may bind phospholipids. They have an additional 20 amino acid extension that affects interactions with co-activating proteins. They are vital for development and metabolism; SF-1 is required for the differentiation of steroidogenic tissues and LRH-1 is involved in the regulation of steroid, bile acid and cholesterol homeostasis

Subgroup 6: The NR6A receptor germ-cell nuclear factor (GCNF) is an orphan receptor that lacks a classic AF-2 domain and acts as a transcriptional repressor through ligand-independent interactions with co-repressors such as octamer-binding transcription factor-4 (OCT4). It has a critical role in development and its activity appears to be controlled by regulation of its gene expression.

For more information on NRs, see the review by Weikum *et al.* (2018). For more information on orphan nuclear receptors, see the review by Mullican *et al.* (2013).

Genes are composed of a number of structural elements (Fig. 1.43), including the 5′ untranslated region (UTR) containing regulatory elements and the promoter (PT), the coding sequences or exons that contain the information for assembling the protein, the non-coding sequences or introns and the 3′ UTRs that are involved in translation termination and post-transcriptional modification to form a mature mRNA. Transcription involves RNA polymerase binding to the PT and generating a primary transcript that is processed into a mature messenger RNA by removal of the introns, addition of the 3′ poly A tail and 5′ capping. This mRNA is subsequently translated into a protein on the ribosomes. Receptors for nuclear hormones act as transcription factors that bind to specific 5′ regulatory regions to regulate the transcription of target genes.

Steroid hormones diffuse through the plasma membrane of target cells and bind to a specific intracellular SR receptor (Fig. 1.44). SRs for the steroid hormones oestrogens, androgens, progestagens and corticoids are sequestered in the cytoplasm bound to chaperone proteins such as the 90 kDa heat shock protein (Hsp90) in an inactive complex. Binding of the hormone to the receptor induces conformational changes that lead to translocation of the hormone–receptor complex to the nucleus and/or disassociation of the repressive complex and association with co-activators. A dimer of the hormone–receptor complex then interacts via the DNA binding domain with responsive elements (REs) on the 5′ region of specific genes to affect DNA transcription. This exposes template sites on DNA, either directly with co-regulator proteins and chromatin remodellers or by influencing pre-existing

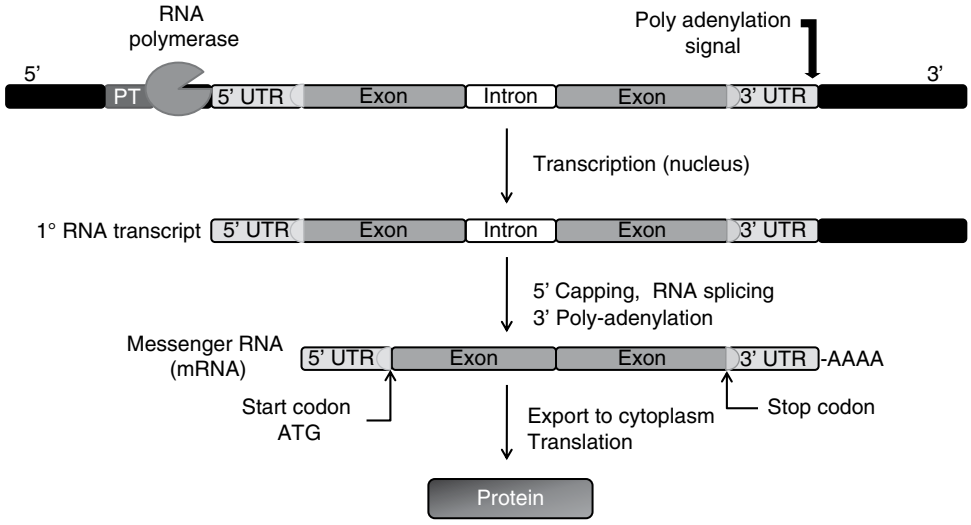


Fig. 1.43. The process of gene transcription and translation.

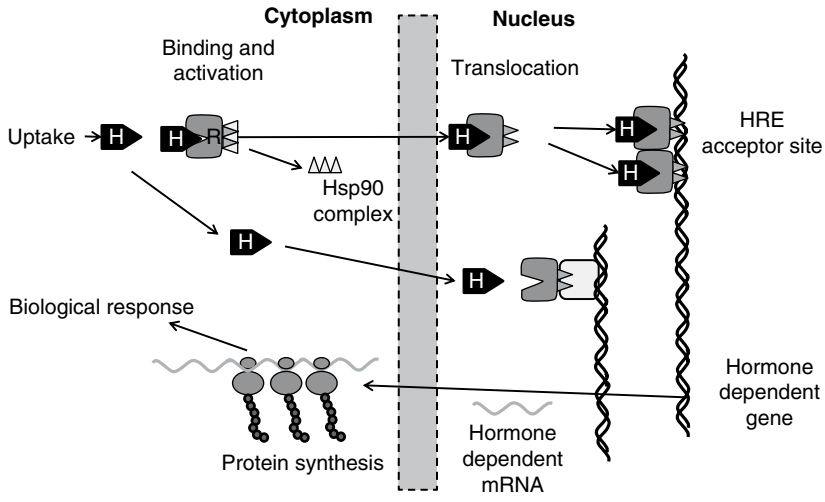


Fig. 1.44. Mechanism of action of intracellular receptors

repressor molecules, to increase the initiation sites for RNA polymerase and increase transcription. Other NRs are retained in the nucleus and are constantly bound to their recognition sites on DNA, with their activity normally repressed by binding to co-repressors. Ligand binding triggers the recruitment of co-activators and the subsequent activation of gene expression. Receptors of this type, such as RAR and LXR, form heterodimers with RXR on direct repeat DNA REs, while the VDR forms homodimers. LRH-1 and SF-1 bind to DNA as a monomer and recognize extended half-sites within REs.

NRs modulate transcription to either activate or repress expression of specific genes. When ligand binding activates the receptor, co-activator proteins are recruited which initiate the formation of large protein complexes containing histone modifying enzymes such as histone acetyltransferases (HATs) or histone methyltransferases (HMTs). These activities facilitate the opening of chromatin, making it accessible to additional regulatory proteins, so the

general transcriptional machinery and RNA polymerase II are able to drive transcription (see discussion below on chromatin structure). NRs can also repress transcription by binding to co-repressors in their inactive state, as seen with receptors that are retained in the nucleus. These co-repressor proteins recruit histone-modifying enzymes such as histone deacetylases (HDACs), which compact the chromatin and block the transcriptional machinery from accessing the DNA. NRs can also interact with negative DNA response elements, which results in co-repressor recruitment to block transcription. This affects de novo mRNA and protein synthesis, which alters the metabolism and function of the target cell. These actions of steroid hormones that affect gene expression take a much longer time frame (hours) to occur compared with changes in activity of proteins due to phosphorylation, which can occur in a few minutes. Actinomycin D (an inhibitor of RNA transcription) and puromycin (an inhibitor of protein synthesis) can be used to inhibit these effects of steroid hormones.

Steroid hormones can also affect the extent of mRNA degradation. In order to separate the effects of RNA synthesis from degradation, a nuclear run-on assay can be used to measure the rate of mRNA synthesis. This involves isolating intact nuclei from cells treated with hormone, allowing transcription to finish and then comparing the amount of gene-specific RNA synthesized compared with an untreated control preparation.

Structural and functional domains of nuclear receptors

Different nuclear receptors have common structural domains involved in hormone (ligand) binding to the receptor, binding of the hormone–receptor complex to DNA and gene activation to stimulate gene transcription (Fig. 1.45). Other regions are involved in dimerization of the receptor and translocation of the receptor to the nucleus.

N-TERMINAL DOMAIN (NTD) The NTD is a highly disordered domain, containing the activation function-1 region (AF-1), which interacts with a variety of co-regulatory proteins in a cell. The NTD is the target for numerous post-translational modifications, including phosphorylation, SUMOylation (small ubiquitin-like modifier proteins) and acetylation.

DNA-BINDING DOMAIN (DBD) This region of the receptor comprises 60–70 amino acids and is the most conserved among all the nuclear receptor domains. This region is where the hormone–receptor complex binds to the response element (RE) of hormone-responsive

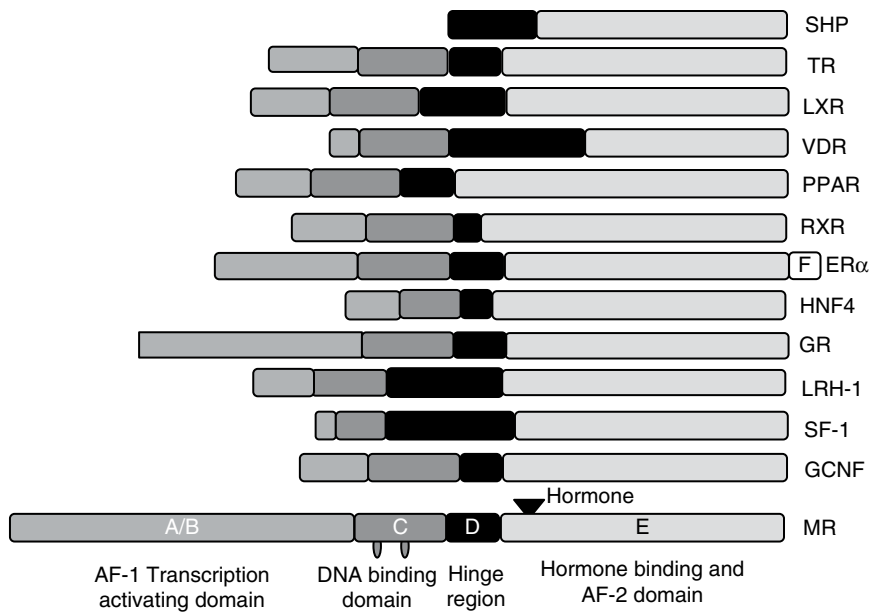


Fig. 1.45. Structural domains of intracellular receptors.

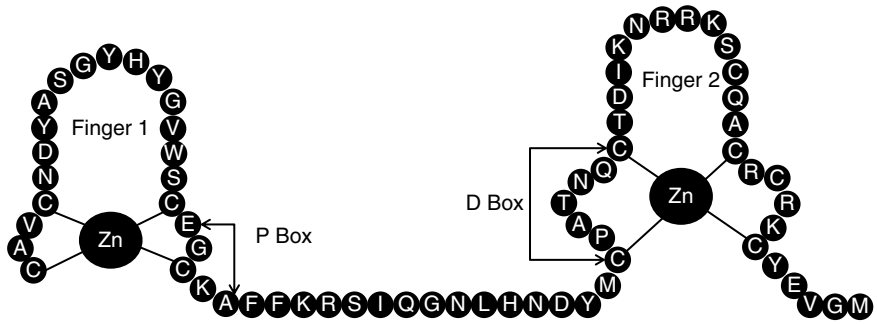
genes to regulate transcription (Fig. 1.46). The DBD has two subdomains that each contains four cysteine residues that coordinate to a zinc ion to create the DNA-binding 'zinc finger' motif. The first zinc finger contains the P box, which recognizes the RE for that receptor on the hormone-responsive gene. The residues in the P box determine which type of RE consensus sequence is recognized by the receptor. The second zinc finger makes non-specific contacts with the DNA backbone. The peptide loop in this subdomain contains the distal box, or 'D box', that contains residues for receptor dimerization. The zinc finger is a common amino acid motif found in transcription factor proteins that bind to specific DNA sequences, with the amino acids at the contact site defining the DNA sequence that is recognized by the zinc fingers.

HINGE REGION The hinge region is a short, flexible linker between the DBD and the LBD. This region has the least sequence and size conservation among the nuclear receptors.

LIGAND-BINDING DOMAIN (LBD) The ligand-binding domain (LBD) is the region where the hormone binds to the receptor. The sequence in this region determines the specificity of the receptor for the hormone. It contains a hydrophobic ligand-binding pocket (LBP) sized to accept its ligand which has polar residues that make key hydrogen bonds with the ligand to hold it in the correct orientation. The LBD not only binds to ligands but also interacts directly with co-regulator proteins via the activation function-2 region (AF-2), which, upon ligand binding, facilitates interaction with different co-regulator proteins.

Nuclear Receptor–DNA Interactions

Nuclear receptor DBDs bind to a variety of REs whose nucleotide sequences can take the form of an inverted palindrome, direct repeat, or extended monomeric sites. The identification of REs in the 5′ region of a gene suggests that NRs regulate the expression of that gene. Response elements for steroid hormone receptors (HREs) have a similar structure consisting of pairs of hexamers arranged as inverted palindromes or direct repeats separated by a defined number of intervening bases. The steroid receptors (GR, MR, AR and PR) bind to RE 5′-AGAACA-3′, while ER binds to 5′-AGGTCA-3′. The HREs are arranged as homodimers with the axis of symmetry over the



Receptor	P box AA sequence
ER	EGCKA
TR	EGCKG
RAR	EGCKG
GR	GSCKV
PR	GSCKV

Fig. 1.46. Zinc fingers in DNA-binding domains of intracellular receptors. The P box sequences define the specificity of receptor binding to DNA.

middle of the palindrome and separated by a spacer region that varies in length, with the most common spacer being 3 bp (Fig. 1.47). The length of this spacer allosterically affects the rate of transcription after receptor binding. Receptors for TR, VDR, PPAR, LXR, PXR and FXR bind to RE 5′-AGGTCA-3′ which are arranged as direct repeats, as heterodimers along with the RXR receptor (named for binding to 9-*cis* retinoic acid), with the two sites separated by a spacer sequence from 0 to 5 bp long. The polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) can bind to the RXR receptor *in vivo*, which may explain the vital role of DHA in development. LRH-1 and SF-1 are examples of receptors that bind extended monomeric sequences. These REs contain one AGGTCA site as well an A/T-rich sequence directly upstream.

The sequence of the zinc finger motif in the receptor and the structure of the REs explain the precise interaction between proteins and DNA that is necessary for the regulation of gene transcription. The amino acids in the zinc finger domain recognize three base pairs on DNA, and it is possible to change these amino acids so that the protein will bind a different DNA sequence. Protein modules containing multiple zinc-finger motifs have been engineered. This system can potentially be exploited in gene therapy to specifically induce a transgene containing specific response elements. These modules have also been fused with an endonuclease to create a sequence-dependent endonuclease to introduce breaks at specific sites of genomic DNA for gene editing (zinc finger nuclease, see Section 2.4).

For many proteins, different variants are formed by differential splicing of exons during processing of the mRNA. Splice variants have been identified for several nuclear receptors and some of these are truncated proteins that lack a functional AF-2 domain, which is required for activation and nuclear translocation. These can act as dominant negative repressors of receptor activity by competing with the wild-type receptor for binding with ligands and other components of the signalling cascade and not stimulating gene transcription. Other variants can act in a dominant positive manner to enhance the activity of the wild-type receptor. This is yet another level of regulation of endocrine systems. For example, the mechanism of dominant negative activity of G protein has been described (Marivin *et al*., 2016).

Organization of nuclear chromatin

CHROMATIN STRUCTURE The DNA in eukaryotic cells is tightly associated with protein in the form of chromatin (Fig. 1.48). The structure of chromatin affects several biological processes, including DNA replication, DNA repair, cell division and transcription. Chromatin exists as a string of nucleosomes, which are composed of 146 bp of DNA wrapped around a core of eight histones. Histones are small basic proteins that are rich in arginine, lysine and histidine. The histone core of the nucleosome consists of two each of H2A, H2B, H3 and H4 and this complex is coated with positive charges that interact with the negatively charged DNA. Histones H3 and H4 are very similar between species, with a difference of only two amino acids in H4 and four amino acids in H3 found between pea and calf thymus. An additional histone (H1) binds the linker DNA between two nucleosomes and is involved in the packaging of the individual nucleosomes to form condensed chromatin. There are no changes in the histones that are related to cell function and they are not involved in binding of the hormone–receptor complex to chromatin.

Disruption of the local and higher-order nucleosome structure is required for proper access of DNA sequences by different nuclear proteins. This disruption can be either transient unwrapping of

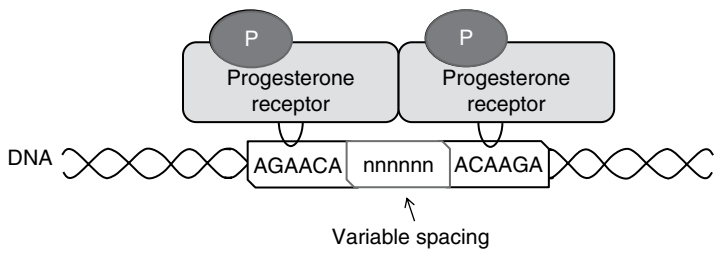


Fig. 1.47. Binding of receptor to the hormone response element (HRE).

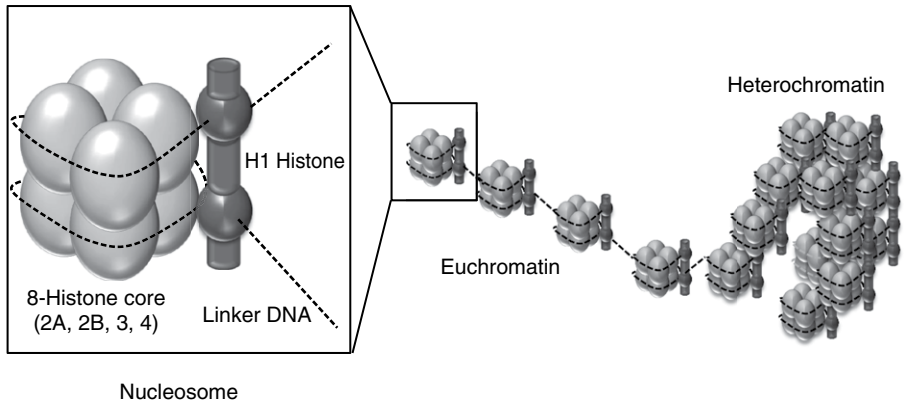


Fig. 1.48. Nucleosome structure of chromatin.

DNA followed by re-wrapping at the same entry/exit point or migration of the nucleosome complex along the DNA by simultaneous unwrapping and re-wrapping with a new sequence.

DNA that is highly condensed (heterochromatin) is often inactive, with transcription taking place in the more loosely packed regions of the genome (euchromatin), which can be detected by their hypersensitivity to nucleases or chemical probes. The histones in active chromatin are highly acetylated (by the enzyme histone acetyltransferase (HAT)), which removes a positive charge on lysine residues and reduces the binding of histones to DNA and the packing density of the chromatin. The binding of the hormone–receptor complex produces hypersensitive sites in chromatin, which allows the binding of other transcription factors to initiate gene transcription. Conversely, deacetylation (by histone deacetylase (HDAC)) promotes nucleosome condensation and decreased transcription. A number of inhibitors of HDAC (e.g. Scriptaid (N-Hydroxy-1,3-dioxo-1H-benz[de]isoquinoline-2(3H)-hexanamide)) have been developed and are useful to maintain open chromatin structure to increase gene transcription (see Section 2.4).

Histone methylation (by histone methyltransferases) can either activate or repress gene transcription, depending on the residue modified and the number of methyl groups incorporated. Methylated amino acids are recognized by enzymes that influence transcription. Histones can also be modified by phosphorylation or ubiquitination.

Chromatin also contains non-histone proteins or acidic proteins, which are rich in aspartic acid and glutamic acid. Many types of acidic proteins are present and their composition varies between tissues. One group of these, the non-histone high-mobility group (HMG) proteins, is the second most abundant protein next to histones and consists of three families: HMGA, HMGB and HMGN. HMGA proteins contain AT-hooks (i.e. adenine-thymine hooks), nine amino acid segments that bind AT-rich DNA stretches in the minor groove. HMGB proteins contain HMG-boxes, 80 amino acid domains that bind into the minor groove of DNA with limited or no sequence specificity. HMGN proteins bind inside nucleosomes, between the DNA and the histone octamer.

HMG proteins can modify chromatin structure to allow binding of transcription factors at the promoter and enhancer sequences. They have been implicated in the regulation of transcription by enhancing the binding of steroid hormone receptors to their target sites on DNA. In particular, HMGB1 and HMGB2 bind to specific DNA structures and they can also modify and stabilize the structure of DNA to aid in the assembly of protein complexes at the promoter. They thus act to increase the sequence-specific binding of steroid hormone receptors to DNA and increase transcription of steroid hormone-responsive genes. However, HMG proteins do not stimulate binding of other nuclear receptors, such as VDR, RAR and RXR.

Methylation of the DNA sequence itself, primarily at CpG sites (cytosine followed by a guanine base in the DNA sequence), alters the extent of chromatin condensation and the binding and activity of transcription factors that can affect local gene transcription activity. These changes, along with modifications to histones, can be heritable and these are termed epigenetic changes. Epigenetic

changes can occur from different environmental exposures (smoking, rearing environment) and are a mechanism for the biological embedding of experience. We now consider gene × epigenetics × environment interaction when studying the genetic control of physiological functions.

NUCLEAR MATRIX The presence of a structural framework of the nucleus, a three-dimensional skeletal network of ribonucleoproteins called the nuclear matrix, has been proposed. The matrix was thought to have a structural and organizational role to allow the nucleus to be organized into different domains for DNA replication, transcription and RNA splicing. This is analogous to the cytoskeleton, which is involved in the intracellular transport of molecules in the cytosol and trafficking of components between cellular compartments. The nuclear matrix proteins have been identified by extracting nuclei with Triton X-100 detergent, followed by treatment with DNase 1 and 2 M NaCl. However, the composition of the nuclear matrix varied widely, depending on the extraction methods used. Thus, the *in vivo* existence of this structure has been widely debated and the presence of a filamentous structure within the nuclei of living cells that is similar to the cytoskeleton has not been conclusively demonstrated. If there is no internal nuclear matrix, it is likely that the folded chromosomes and their associated proteins provide a structural basis for nuclear compartmentalization. The different conformations of chromatin can dramatically affect gene expression activity.

When the hormone–receptor complex binds to DNA, it recruits and interacts with other transcription factors that can modify the local chromatin structure, which in turn modulates the recruitment and activity of RNA polymerases to repress or enhance transcription. The increase in initiation sites caused by binding of steroid hormone–receptor complexes to DNA can be demonstrated using inhibitors of free RNA polymerase, such as rifampicin or α-amanitin. In this experiment (Fig. 1.49), RNA polymerase is added to chromatin, which then binds to available initiation sites. Rifampicin is then added, which binds to and inhibits the excess RNA polymerase not bound to chromatin. Nucleotides are then added to start transcription and, after one copy is made, RNA polymerase is inhibited. The number of copies of RNA transcribed is a measure of the number of initiation sites on the chromatin.

For further details on HMG proteins, see Mallik *et al.* (2018); for chromatin structure see Biddie and John (2014); for more information on the composition of the nuclear proteins see Sureka and Mishra (2021) and Razin *et al.* (2014); and for epigenetics see Aristizabal *et al.* (2020).

Identification of DNA regulatory sequences and binding proteins

If the 5′ sequence of the gene containing the regulatory elements is known, it can be scanned to locate

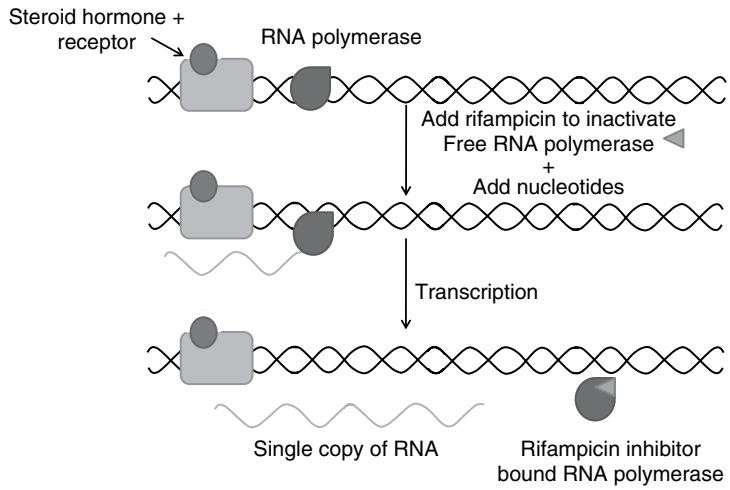


Fig. 1.49. Experiment to illustrate that steroid hormone receptor binding increases RNA polymerase initiation sites in target genes.

a known 'consensus sequence' for binding sites of transcription factors and other regulators of gene expression. This can be accomplished using a basic local alignment search tool (BLAST) at http://ncbi.nih.gov/BLAST/. The sequence can also be examined for the presence of consensus binding sequences for transcription factors using software such as MatInspector (http://www.genomatix.de) described in Cartharius *et al.* (2005).

There are several experimental techniques that can be used to identify regions of the DNA where proteins bind, the identity of the binding protein and the role of these regions in regulating gene expression. DNA-binding proteins and their binding sites on DNA can be identified by DNase footprinting, chromatin immunoprecipitation and sequencing (ChIP-seq) analysis and electrophoretic mobility shift assays (EMSAs). DNA regulatory sequences can be identified using gene reporter assays. These techniques can be also used to confirm the nucleotide sequences that are involved in hormone–receptor binding to DNA.

DNase footprinting assays (Fig. 1.50) begin by labelling a double-stranded DNA sequence that contains the region of DNA (e.g. a promoter) that is suspected of binding protein factors. The labelled DNA is then incubated with a source of binding protein, such as an extract of nuclei from cells in which the gene promoter functions or a purified NR preparation. A control incubation is also carried out using a source of protein that does not bind the DNA. The protein–DNA complex is then subjected to limited digestion with DNase I to introduce a small number of nicks in each DNA molecule. The DNA fragments produced are then separated by size, using denaturing polyacrylamide gel electrophoresis to generate a 'ladder' of DNA fragments. The region of the DNA probe bound by

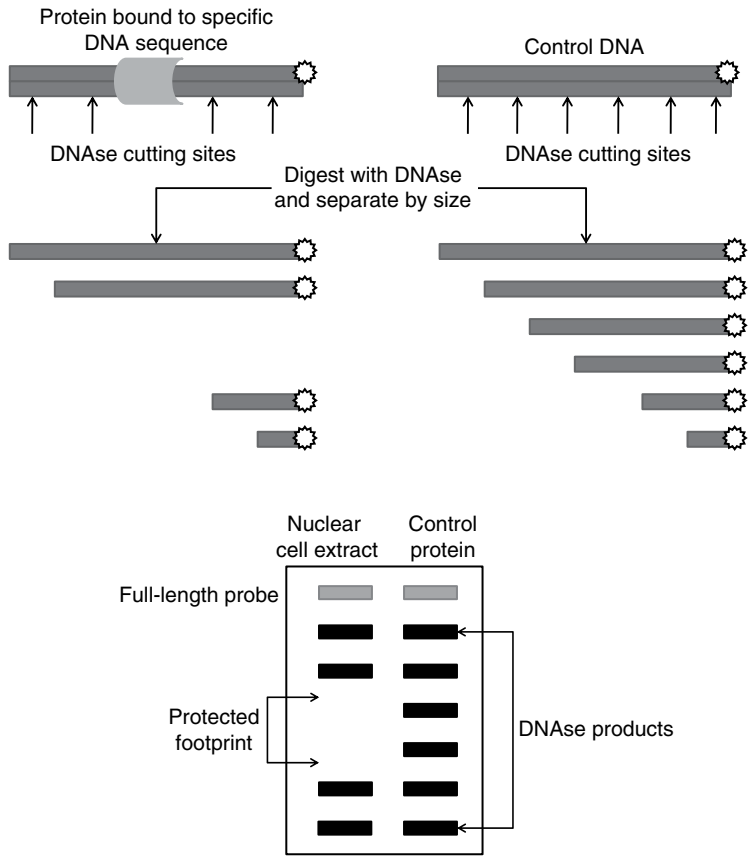


Fig. 1.50. DNase footprinting assay.

the protein is protected from DNase digestion and this results in a hole or 'footprint' in the ladder compared with the control incubation. This region of the DNA can then be isolated and sequenced.

Another useful technique for identifying transcription factor binding elements in DNA and characterizing their involvement in gene regulation is chromatin immunoprecipitation and sequencing (ChIPseq) analysis (Fig. 1.51). ChIPseq produces a library of target DNA sites that bind to a protein of interest and is a powerful method to selectively identify DNA sequences bound by a particular protein in living cells. Tissue that is actively transcribing the genes of interest is treated with formaldehyde to cross-link the protein transcription factors to the DNA-binding sites that they occupy. The chromatin is then isolated, sheared into small fragments and treated with an antibody against the transcription factor of interest. This immunoprecipitates the chromatin fragments containing the bound transcription factor. The cross-linking is then reversed and the DNA is purified from the precipitated chromatin. Polymerase chain reaction (PCR) is then used to amplify specific DNA sequences in the gene of interest to see if they were precipitated with the antibody. Oligonucleotide adaptors are added to the small stretches of DNA that were bound to the protein of interest to enable large-scale parallel sequencing. The sequences can then be identified and localized to the gene or region to where the protein was bound.

The electrophoretic mobility shift assay (EMSA) (Fig. 1.52) is used to confirm that a small region of DNA binds to nuclear proteins. A DNA fragment suspected of binding the protein is labelled and incubated with a source of binding protein, such as a nuclear extract, and the mixture is separated by non-denaturing gel electrophoresis to preserve DNA–protein complexes. The unbound DNA probe has a low molecular weight and moves rapidly through the gel, while the DNA–protein complex is of much larger size and is 'shifted' to a higher region of the gel. The specificity of binding of protein to the labelled probe is confirmed by adding an excess of the same DNA but is unlabelled to the assay mixture. The binding of the unlabelled DNA should result in decreasing the intensity of the labelled higher-molecular-weight band. Doing a similar competition experiment but using an unrelated oligonucleotide should have no effect on the intensity of the labelled higher-molecular-weight band. To confirm the identity of the protein that is bound to the DNA, a specific antibody against the protein is included to specifically 'supershift' the band to a higher molecular weight due to binding of the protein by the antibody.

DNA sequences that are important in regulating gene expression can also be identified using an *in vitro* gene reporter assay (Fig. 1.53). The experimental method involves the following.

1. Linking the regulatory sequences of interest to a test or reporter gene that is easy to measure, such

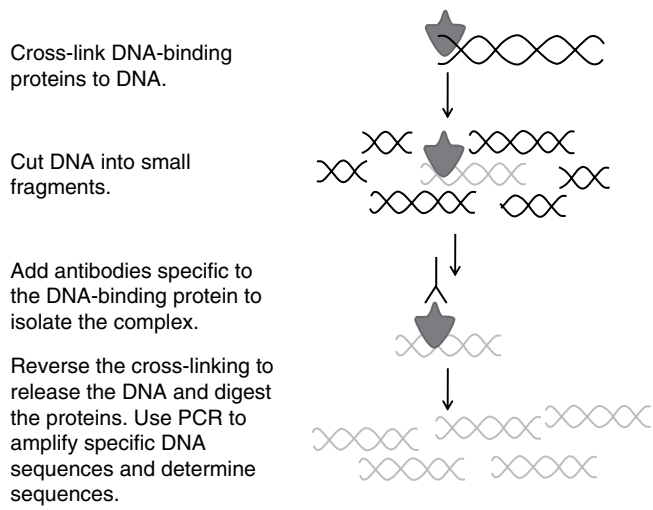


Fig. 1.51. Chromatin immunoprecipitation and sequencing (ChIPseq) analysis.

Loaded compounds	1	2	3	4	5
Labelled DNA	+	+	+	+	+
Nuclear extract	–	+	+	+	+
Excess unlabelled DNA	–	–	+	–	–
Unrelated oligonucleotide	–	–	–	+	–
Specific antibody	–	–	–	–	+

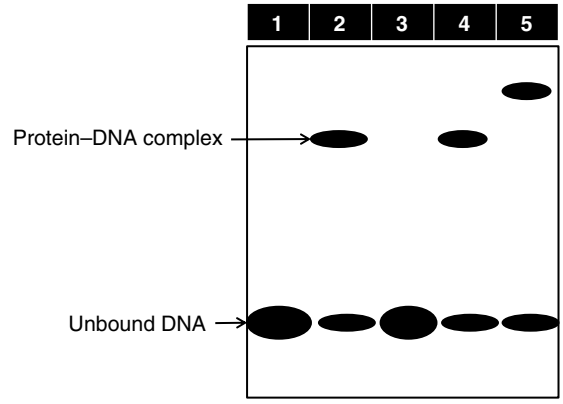


Fig. 1.52. Electrophoretic mobility shift assay.

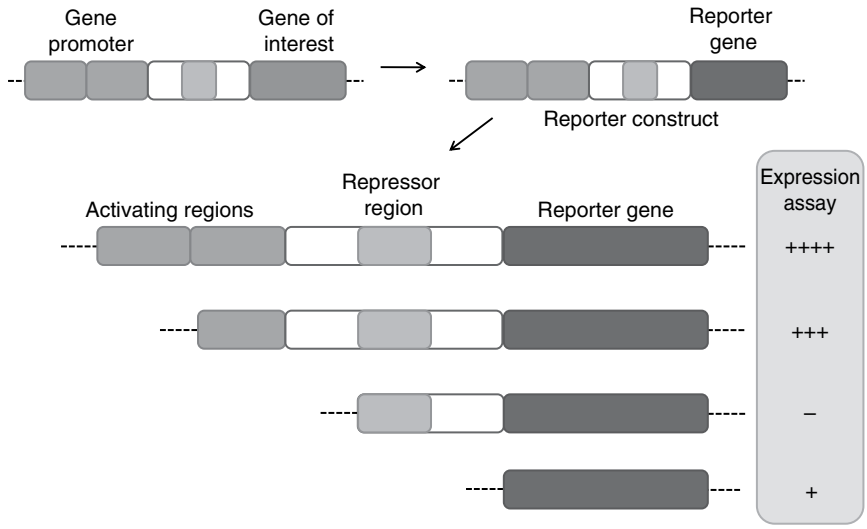


Fig. 1.53. Gene reporter assay for identifying regulatory elements in genes.

as firefly luciferase (see Table 2.7 for other reporter genes) and transfecting the gene construct into a cell line. For assessing hormone-responsive elements, the cell line must also contain the receptor for the hormone.

2. The cells are then treated with the hormone and the activity of the test gene is measured.
3. Serial deletions and/or point mutations are made in the sequences of interest and the activity of the reporter gene is measured after hormone treatment.

The changes in activity will identify which specific regions are responsible for the activation or repression of the test gene. Deletion of sequences important in activation of the gene by a hormone–receptor complex will eliminate the stimulation of gene transcription by the hormone. Deletion of repressor regions will result in increased activity of the reporter gene.

Integration of peptide and steroid hormone actions

The classic descriptions of peptide and steroid hormone actions described above are not the whole story. Steroid hormones can also cause rapid effects that occur within a few minutes and are too rapid to be mediated by increases in transcription and translation. The mechanism of these non-genomic effects may involve either a subpopulation of the classical steroid hormone receptors that are localized outside the nucleus, or separate unrelated cell membrane receptors for steroids. Classical steroid hormone receptors have been localized to the cell membrane using imaging or cell fractionation methods. Some synthetic ligands that specifically activate either the genomic or the non-genomic responses of steroids, particularly oestrogen and vitamin D, have been developed. Some important genes are regulated exclusively by membrane steroid hormone receptors and do not involve the nuclear receptor pool. Alternatively, extra-nuclear receptors can engage in crosstalk with nuclear receptor pools to collectively regulate physiological and pathological processes.

The extra-nuclear receptors are often the same proteins as the nuclear receptors, as occurs for cell membrane-localized oestrogen, progesterone, androgen, glucocorticoid, vitamin D and thyroid hormone receptors. Approximately 5% of the total oestrogen receptor pool is localized at the plasma membrane. This occurs by attachment of palmitic acid to an internal cysteine residue in the LBD of receptor monomers, which promotes the physical association of the receptor with caveolin-1. Caveolin-1 serves as the transport protein that re-localizes the receptor–caveolin-1 complex to caveolae rafts in the plasma membrane. This allows the membrane receptors to activate various G proteins which regulate kinase signals such as ERK and PI3K–AKT, thereby mediating biological processes that impact cell proliferation, survival, migration and many other biological functions.

Non-genomic actions of androgens, oestrogens and progesterone can also be mediated by a subpopulation of receptors in the cytoplasm that interact with various adapters, protein kinases or other signalling molecules (see Section 3.2). Evidence exists for unique cell membrane receptors including a G protein-coupled membrane progesterone receptor (mPR). Progesterone has also been shown to activate an alternative membrane receptor, known as progesterone receptor membrane component 1 (PGRMC1), to affect the growth and viability of various mammalian cells.

The plasma sex hormone-binding globulin (SHBG) is involved in the transport of androgens and oestrogens in the blood and it regulates the amount of free sex steroids that are released to the target cells. In addition, SHBG can bind to a receptor at the cell surface and then bind free steroids, to activate a cAMP second messenger system. In this way, the SHBG may modulate the effects of sex steroids acting on receptors within the cells. Steroids that bind to the SHBG but do not activate the second messenger system act as antagonists. For further information on the rapid non-genomic effects of steroid hormones, see the reviews by Levin (2015) and Levin and Hammes (2016).

Steroid hormones can also affect the activity of protein hormones by stimulating the synthesis of receptors on the cell surface or protein kinases to increase hormone response, or phosphoprotein phosphatases to decrease the response to hormone (Fig. 1.54). As mentioned previously, production of cAMP by the action of protein hormones can also have effects on gene transcription (see Fig. 1.30). This occurs by phosphorylation and activation of the CREB protein by protein kinase A or by modification of the structural proteins in chromatin. Many hormone-responsive genes have specific cAMP-responsive elements in their regulatory regions. Activated CREB protein binds to these regions to activate gene expression.

1.4 Pituitary–Hypothalamic Integration of Hormone Action

Key concepts

- The pituitary gland is linked to and controlled by the hypothalamus, which receives and processes signals from cells such as baroreceptors and osmoreceptors.
- The posterior pituitary is nervous tissue that receives oxytocin and vasopressin from neuroendocrine cells in the hypothalamus; release is by direct nervous stimulation.

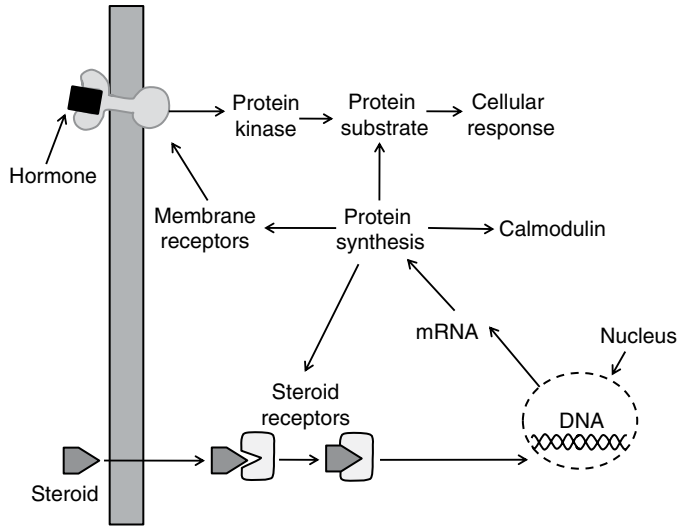


Fig. 1.54. Integration of actions of cell surface and intracellular receptors.

- Oxytocin causes contraction of myoepithelial cells for milk let-down in the mammary gland and in the myometrium for the contraction of the uterus for parturition.
- Vasopressin (antidiuretic hormone) stimulates reabsorption of water from the distal tubular kidney to maintain blood osmolarity.
- The anterior pituitary is glandular tissue, which produces several trophic hormones in specialized cell types that stimulate hormone release by target tissues.
- The anterior pituitary is regulated by releasing hormones and release-inhibiting hormones delivered from the hypothalamus by the hypothalamic–hypophyseal portal system.
- The control of hormone release from the anterior pituitary by the hypothalamus has been demonstrated by a number of experiments.
- Hormone secretion from the pituitary is episodic, regulated by the biological clock in the hypothalamus; secretion is pulsatile due to the pulsatile firing of nerves and release of hypothalamic hormones.
- The biological clock that regulates the circadian rhythm is controlled by a transcription–translation feedback loop (TTFL).
- Endocrine systems are regulated at many levels, including hormone, receptor and downstream signalling pathways.

Structure–function relationship of pituitary and hypothalamus

The hypothalamus is the major integration centre in the brain that participates in the regulation of the autonomic nervous system and most of the endocrine system. The hypothalamus is innervated with many neurons from other parts of the body and receives and processes signals from cells such as baroreceptors and osmoreceptors and other environmental cues. It is located below the third ventricle, above the median eminence in the brain. The pituitary gland or hypophysis is located below the hypothalamus, in a hollow pocket of the sphenoid bone known as the 'sella turcica', and is linked to the hypothalamus via the stalk. Hormone release by the pituitary is under hypothalamic control.

The pituitary gland consists of two distinct lobes: the posterior pituitary or neurohypophysis and the anterior pituitary or adenohypophysis. The posterior pituitary is nervous tissue that develops as an outgrowth of the diencephalon. It receives hormones that are made in neuroendocrine cells in the magnocellular neurons in the hypothalamus and are transported along the axons to the posterior pituitary. The anterior pituitary is glandular tissue and is subdivided into the pars distalis and pars intermedia. It is the 'master gland', which produces several trophic releasing hormones that stimulate hormone release by target tissues. The relationship between the hypothalamus and pituitary is illustrated in Fig. 1.55.

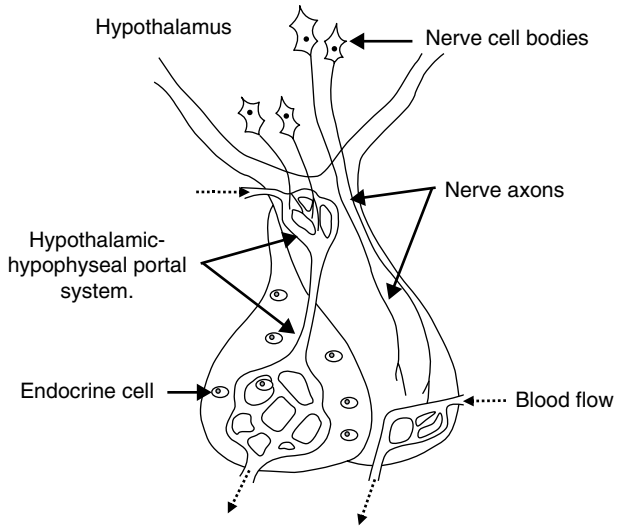


Fig. 1.55. Organization of the pituitary and hypothalamus.

Posterior pituitary hormones

Oxytocin and vasopressin are the two hormones that are released from the posterior pituitary. Oxytocin causes contraction of smooth muscles. These include the myoepithelial cells for milk let-down in the mammary gland, and in the myometrium for the contraction of the uterus for parturition. Vasopressin, also known as antidiuretic hormone (ADH), is the most important regulator of extracellular fluid osmolarity. It stimulates reabsorption of water from the distal tubular kidney to maintain blood osmolarity when blood volume or blood pressure is decreased.

Both vasopressin and oxytocin are polypeptides containing nine amino acids with a disulfide bridge between two cysteines in the molecule (Fig. 1.56). They have a half-life in the plasma of 3–5 min. These two hormones are almost identical, except that, in vasopressin, phenylalanine and arginine replace the isoleucine and leucine of the oxytocin molecule. They are synthesized in the body of hypothalamic neurons as preprohormones with their neurophysin carrier proteins. These are cleaved to active hormones during fast axonal transport from the cell body down the axon in the posterior pituitary and stored in secretory granules at nerve endings that lie on the surfaces of capillaries (Fig. 1.57). Vasopressin and oxytocin are secreted into the capillaries by exocytosis (excretion of substances through the cell membrane) from the neurons that produce them and enter the general circulation.

Release of hormones by the posterior pituitary is under direct nervous stimulation from the hypothalamus in response to changes in osmotic or barometric pressure, pain, fright or stress, adrenal insufficiency, hypoxia or cardiac failure. Oxytocin is also released in response to teat sensory nerve stimulation for the milk ejection reflex.

Anterior pituitary hormones

The pars intermedia is part of the anterior pituitary which is the source of β-endorphins. In mammals, it produces pro-opiomelanocortin (POMC) (see Fig. 1.10) which is cleaved enzymatically into biologically active peptides. These include β-lipotropin (β-LPH) which degrades to β-endorphin, producing analgesia in stress, and melanocyte-stimulating hormone (MSH), which increases skin pigmentation, decreases feed intake and regulates the production of cytokines.

The anterior pituitary produces a number of trophic hormones that cause hormone release from target tissues (Table 1.4). The different hormones are made in specific cells in the anterior pituitary gland, namely thyrotropes (producing TSH), gonadotropes (producing LH and FSH), corticotropes (producing ACTH), melanotropes (producing MSH), somatotropes (producing GH) and mammotropes

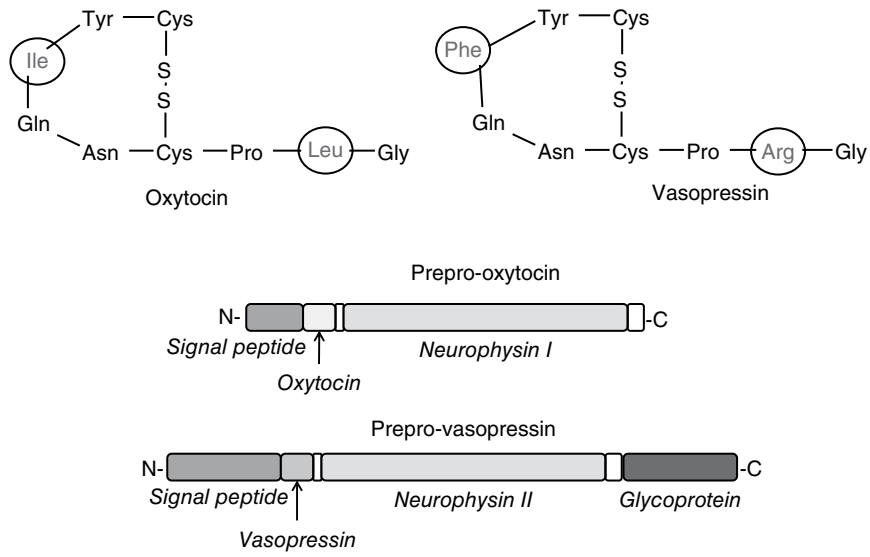


Fig. 1.56. Structures of oxytocin, vasopressin and their preprohormones.

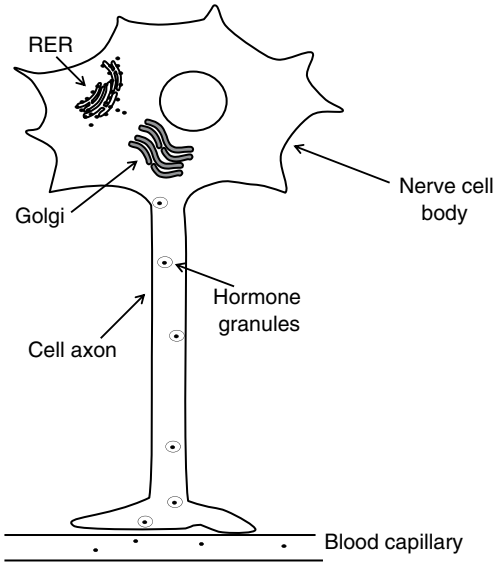


Fig. 1.57. Hormone production and axonal transport by nerve cells. RER, rough endoplasmic reticulum.

(producing PRL). They are released under direct control by releasing hormones and release-inhibiting hormones from the hypothalamus. Hormones of the anterior pituitary are proteins or glycoproteins. They have longer half-lives than the releasing hormones produced by the hypothalamus.

Table 1.4. Hormones of the anterior pituitary.

Hormone	Number of amino acids	Structure
GH	191	Single-chain proteins with two to three disulfide bonds
PRL	199	
TSH	211	Glycoproteins, two subunits with common α subunit
FSH	210	
LH	204	
ACTH	39	Short, linear-chain peptides
MSH	13/22	
β-LPH	91	

Growth hormone (GH or somatotrophin) has both direct effects on metabolism and indirect effects on cell proliferation. The direct effects on metabolism include catabolic effects to increase lipolysis and reduce lipogenesis in adipose tissue, and anabolic effects to increase amino acid uptake and promote synthesis of protein. The indirect effects are mediated by synthesis of insulin-like growth factor 1 (IGF-1 or somatomedin) and its binding proteins in the liver and peripheral tissues. IGF-1 stimulates the growth of chondrocytes (cartilage cells), to increase bone growth, and satellite cells in muscle, to increase muscle fibre growth, and stimulates amino acid uptake and protein synthesis.

Thyroid-stimulating hormone (TSH) stimulates the endocytosis of iodinated thyroglobulin by the

follicular cells in the thyroid gland to increase the synthesis and release of thyroid hormones (see Section 1.2).

Adrenocorticotrophic hormone (ACTH) stimulates the mobilization of cholesterol in the mitochondria of the adrenal cortex, which provides more substrate for the side-chain cleavage cytochrome P450 (CYP11A1) to increase the production and release of corticosteroids.

Luteinizing hormone (LH) stimulates testosterone production by Leydig cells in the testis of males and drives ovulation in females. FSH stimulates secretion of inhibin by Sertoli cells to regulate spermatogenesis in males, while in females it drives the development of follicles and secretion of oestradiol.

Prolactin (PRL) stimulates the synthesis of the milk proteins casein and lactalbumin (see Section 4.1). In poultry, it is responsible for the initiation and maintenance of incubation behaviour or broodiness (see Section 6.1).

Hypothalamic releasing and release-inhibiting hormones

The release of hormones from the anterior pituitary is controlled by releasing hormones and release-inhibiting hormones produced by specific neurons in the hypothalamus. The hypothalamic hormones are delivered from the hypothalamus to the anterior pituitary by the hypothalamic–hypophyseal portal system (see Fig. 1.55). The endocrine cells of the anterior pituitary then release their trophic hormones into a second capillary network to enter the systemic circulation. These trophic hormones cause the target endocrine tissue to release the ultimate hormone to produce systemic effects (Fig. 1.58).

The control of hormone release from the anterior pituitary by the hypothalamus has been demonstrated by a number of experiments. These include the following.

1. Placing a mechanical barrier between the hypothalamus and the pituitary, or ectopic transplantation of the pituitary gland (to another site that has a good blood supply, see Section 2.1) will either decrease or enhance secretion of certain pituitary hormones (depending on whether that hormone is controlled by releasing hormones or release-inhibiting hormones).
2. Use of pharmaceutical agents that affect neurotransmitter production, or electrical stimulation of discrete hypothalamic sites, can affect the release of pituitary hormones.
3. Hypothalamic extracts injected into an intact animal affect pituitary secretions.
4. Releasing hormones have been localized to specific hypothalamic neurons using labelled antibodies by immunocytochemistry (see Section 2.1).

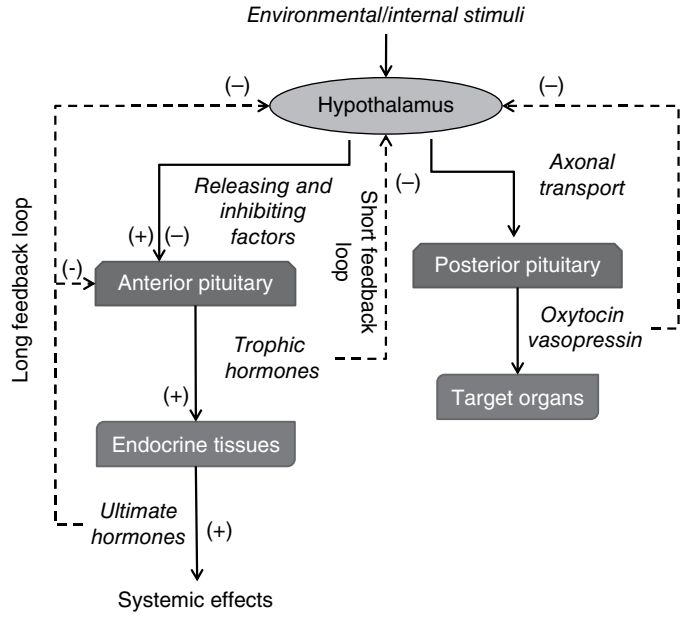


Fig. 1.58. Regulation of pituitary hormone release.

5. Levels of these releasing and release-inhibiting hormones are higher in the hypothalamic–pituitary portal system than in the systemic circulation.
6. Generating antibodies to hypothalamic releasing hormones will inhibit pituitary hormone secretion (for example, see immunization against GnRH in Section 3.3).

The function of the releasing and release-inhibiting hormones from the hypothalamus is to control the secretion of the anterior pituitary hormones. For each type of anterior pituitary hormone there is usually a corresponding hypothalamic releasing hormone. Some hormones, namely GH, MSH, PRL and the gonadotrophins, are regulated by both releasing hormones and release-inhibiting hormones (Table 1.5). This suggests that these hormones may be under a finer level of control than other anterior pituitary hormones.

The hypothalamic releasing hormones are generally made as a larger prohormone, which is cleaved later to form active peptide hormones. They are all simple peptides or proteins, ranging in size from three amino acids for thyrotropin-releasing hormone (TRH, pyro-E-H-P-amide), ten amino acids for gonadotrophin-releasing hormone (GnRH, pyro-E-H-W-S-Y-G-L-R-P-G-amide), 12 amino acids for gonadotrophin release-inhibiting hormone (GnIH), 14 amino acids for growth hormone release-inhibiting hormone (GHIH), 41 amino acids for corticotrophin-releasing hormone (CRH) and 44 amino acids for growth hormone-releasing hormone (GHRH).

The overall scheme of the regulation of metabolic hormones is given in Fig. 1.59 and the regulation of reproductive hormones is given in Fig. 1.60.

Control of hormone release

Hypothalamic neurons receive inputs from a number of sources, including the higher brain centre and emotions, a number of exterior, environmental and social stimuli, internal rhythms, indicators of metabolic state such as temperature, energy level and osmolarity, and from endogenous hormones by feedback. Release of hormones by the posterior pituitary is under direct nervous stimulation from the hypothalamus. Nervous stimulus in response to environmental or internal signals also triggers the release of releasing hormones and release-inhibiting hormones from the hypothalamus which regulate hormone release by the anterior pituitary. The releasing hormone precursors are made in cell bodies and transported down the axons to the nerve endings for storage. They are released by exocytosis of granule contents into the hypothalamic–pituitary portal blood system to be delivered to the anterior pituitary in response to electrical signals from other

Table 1.5. Hypothalamic control of anterior pituitary hormones.

Releasing hormone	Trophic hormone	Target tissue and hormone	Ultimate effect
TRH (thyrotrophin-releasing hormone)	TSH (thyroid-stimulating hormone)	↑ T_4 and T_3 from thyroid	Regulates metabolic rate
	PRL (prolactin)	Mammary gland	Synthesis of milk
GnRH and GnIH (gonadotrophin-releasing and inhibiting hormone)	LH (luteinizing hormone)	Ovary/testis	Affects ovulation/testosterone production
	FSH (follicle-stimulating hormone)	Ovary/testis	Affects gonadal development
CRH (corticotrophin-releasing hormone)	ACTH (adrenocorticotrophic hormone)	↑ Adrenocortical steroids from adrenal cortex	Response to stress
	β-LPH (β-lipotrophin degrades to β-endorphin		Analgesia in stress neurotransmitter
GHRH and GHIH (growth hormone-releasing and inhibiting hormones)	GH (somatotrophin, ST)	↑ Somatomedin (IGF-1) production by liver	Increased growth
		Adipose and muscle	↓ Lipid, ↑ muscle protein
MRF, MIH (melanocyte-stimulating hormone release and inhibiting hormones)	MSH (melanocyte-stimulating hormone)	Melanocytes	↑ Skin pigmentation
		CNS	↓ Feed intake
		Immune system	Anti-inflammatory response
PRH and PIH (prolactin-releasing and -inhibiting hormones)	PRL (prolactin)	Mammary gland	Synthesis of milk

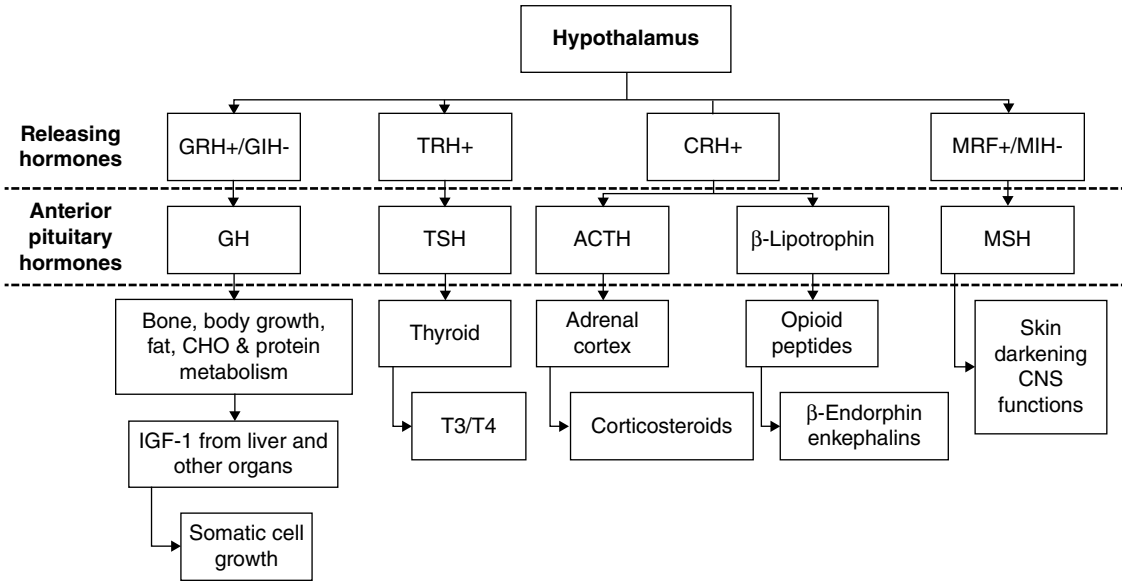


Fig. 1.59. Overview of regulation of metabolic hormones.

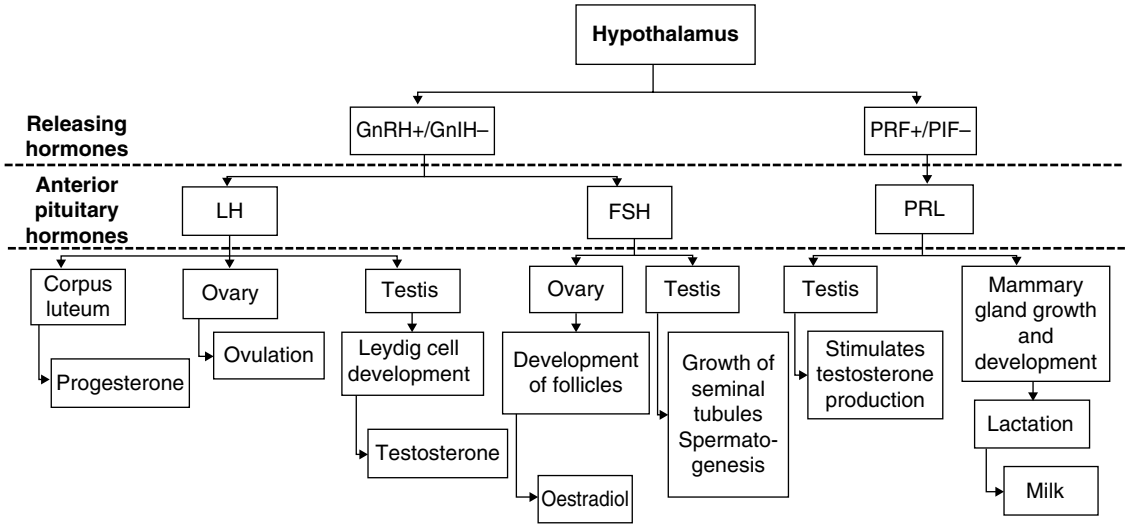


Fig. 1.60. Overview of regulation of reproductive hormones.

neurons. The hypothalamic hormones are released in nanogram quantities and this causes the release of trophic hormone by the anterior pituitary in microgram amounts. The trophic hormone then causes release of milligram amounts of the ultimate hormone by the target gland. Note that each step in this process produces an amplification of the response (Fig. 1.61).

Many biological processes display an endogenous circadian rhythm of about 24 h ('circadian', from the Latin words *circa* meaning 'around' and *dies* meaning 'day') that allows organisms to adapt to changes in their environment over time and thus enhance their survival. Circadian rhythms are conserved throughout evolution and exist in life forms from unicellular cyanobacteria and protozoans to

all multicellular organisms, including fungi, plants, insects, rodents and humans. The circadian system consists of a self-sustained 24 h rhythm generator that is entrained by external cues, called zeitgebers (from German 'time givers'), which include external light sensed by the ganglion cells of the retina, as well as temperature and redox cycles to allow the optimal scheduling of physiological processes.

The 2017 Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young 'for their discoveries of molecular mechanisms controlling the circadian rhythm'. They and others have shown that the biological clock is regulated by a transcription–translation feedback loop (TTFL), which is a complex network of reactions involving regulated transcription, protein phosphorylation and degradation of TTFL components, protein complex assembly, nuclear translocation and other post-translational modifications, which generate oscillations with a period of about 24 h.

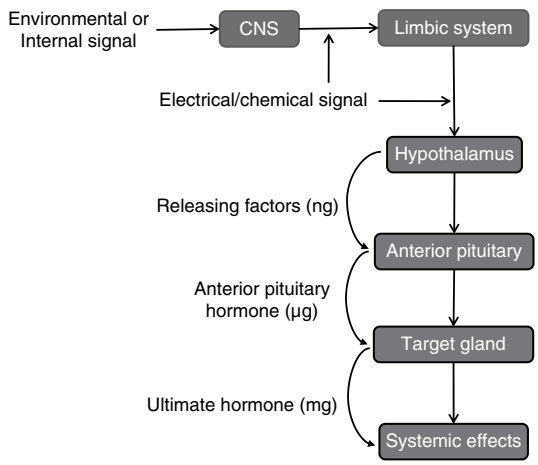


Fig. 1.61. Amplification of endocrine signal response.

In the TTFL (Fig. 1.62), the transcription factors CLOCK and BMAL1 form a heterodimer and bind to a specific DNA sequence called E-box to activate the expression of cryptochrome (CRY) and period (PER) proteins. These proteins heterodimerize and translocate to the nucleus to repress the transcriptional activity of CLOCK and BMAL1 in a negative feedback loop. The PER and CRY proteins accumulate during the night and are gradually degraded during the day through post-transcriptional modifications such as phosphorylation and ubiquitination. When levels of CRY and PER decrease, the CLOCK–BMAL1 can then restart the transcription of PER and CRY to complete the TTFL. This results in protein levels that oscillate over a 24 h cycle, in synchrony with the circadian rhythm. CLOCK–BMAL1 also induce the expression of the NR1D1/2 nuclear receptors which act in a secondary loop with ROR nuclear receptors to regulate the cyclic expression of clock-controlled genes that regulate circadian physiological functions. This system is further regulated by kinases CK1δ/ε and ubiquitin ligases that control the activity and stability of the clock proteins.

Circadian rhythms are regulated centrally by the biological clock in the suprachiasmatic nucleus of the hypothalamus which anticipates day/night cycles to control physiological outputs such as sleep patterns, behaviour, body temperature, hormone release, blood pressure and metabolism. A number of hormones are released in a circadian pattern to regulate metabolism according to circadian rhythms. For example, the levels of cortisol are highest in the morning and decrease in the

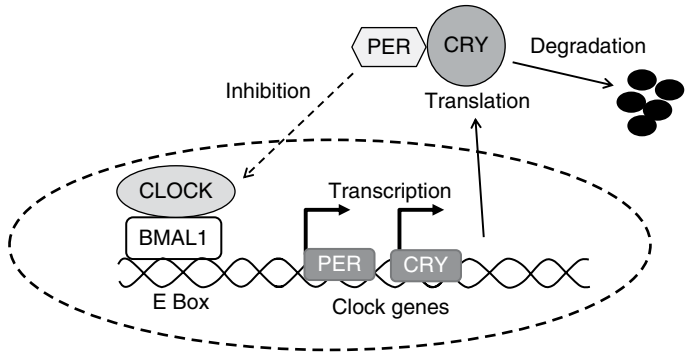


Fig. 1.62. The transcription–translation feedback loop of the circadian clock.

afternoon and evening. Levels of leptin and growth hormone increase at night, while both insulin secretion and insulin sensitivity decrease at night. Melatonin acts to synchronize the circadian rhythm and is released during the dark period and decreased by exposure to light.

Clocks also exist in different peripheral tissues, and most organs and tissues can express circadian oscillations in isolation. These peripheral clocks control tissue-specific physiological outputs, such as glucose production, fat storage and release of hormones, and are controlled by environmental cues, including feeding, physical activity and temperature. Examples include the control of glucose homeostasis by the liver and pancreatic clocks and ovulation by the ovarian clock. These peripheral clocks are also regulated by the central clock in the suprachiasmatic nucleus, which regulates circadian rhythms across the entire body via humoral factors and the peripheral autonomic nervous system. This allows the integrated regulation of daily cycles of physiological function and behaviour.

Many hormones are secreted in a pulsatile manner due to the pulsatile firing of nerves and release of hypothalamic hormones. This includes insulin, thyrotropin, TRH, gonadotropin-releasing hormone (GnRH); secretion of somatotrophin is more pulsatile in females than in males (see Fig. 3.17). The pulse frequency, pulse amplitude and average hormone levels can be calculated using a pulse detection algorithm 'Pulsar' developed by Merriam and Wachter in 1982. The nature of this pulsatile release is very important. For example, differences in the frequency of GnRH release by the hypothalamus differentially affect the subsequent release of LH and FSH by the pituitary. In females, the high pulse frequency supports the release of LH better than FSH, while the low pulse frequency supports the synthesis and release of FSH better than LH (see Section 5.1). High pulse frequency of GnRH and an increased frequency and amplitude of LH pulses and elevated LH to FSH ratio, which results in high levels of androgens, are found in human females with polycystic ovary syndrome (PCOS). Pulsatile rhythms of hormone release may sensitize target tissues to the hormone of interest and prevent the down-regulation of receptors that would occur in response to a continuous high level of hormone secretion.

Circulating hormones from the endocrine glands provide negative feedback to both the hypothalamus and the pituitary and this feedback serves to regulate the secretion of hormones. There is a short feedback loop from the anterior pituitary to the hypothalamus and a long feedback loop of the ultimate hormone from the target glands on the central nervous system (CNS), hypothalamus or anterior pituitary (see Fig. 1.58).

Summary: Multiple levels of regulation of endocrine systems

From the material presented in this chapter, it is clear that endocrine systems are regulated at many levels (Table 1.6), including hormone, receptor and downstream signalling pathways.

Table 1.6. Multiple levels of regulation of endocrine systems.

Endocrine component	System
Hormone	Synthesis and storage
	Prohormones and other storage forms
	Control of hormone release
	Binding to carrier proteins
	Delivery to target tissue and activation
	Location specific effect (paracrine and autocrine effects)
	Degradation and elimination
Receptor	Receptor number and binding affinity/specificity
	Recycling of receptors
	Genomic effects and chromatin structure
	Activation versus inhibition pathways
Downstream effects	Concerted, synergistic, non-additive, antagonistic effects
	Effects on protein synthesis and phosphorylation status of proteins
	Multistep amplification of hormone signalling
	Integration of different hormone signals
	Feedback positive and negative

The levels of active hormone are controlled by their rate of synthesis and degradation and whether they are present as inactive prohormones that are activated in the target tissue. Hormone release is controlled by nervous signalling, levels of metabolites and signalling compounds and by trophic hormones. Hormone can be selectively delivered to target tissues or bound to a carrier protein during transport or can have local autocrine or paracrine effects.

Regulation at the receptor level includes the number of receptors present and their binding affinity and specificity for hormones. After binding to hormones, receptors can be degraded or recycled for reuse. Receptors can be coupled to either activation or inactivation pathways for a particular physiological response. Chromatin structure can affect receptor binding and the expression of hormone-responsive genes.

Downstream signalling events that occur subsequent to hormone binding to receptors include concerted, synergistic, non-additive and antagonistic effects on endocrine systems and integration of different hormone signals, for example through the MAPK system. The multiple steps that are involved in hormone signalling and the amount of positive or negative feedback can modify the response. Finally, the products of protein synthesis and phosphorylation status of proteins can affect the endocrine response.

Questions for Study and Discussion

Section 1.1 Introduction

1. What are concerted and non-additive actions of hormones and how can they be explained based on their mechanism of action?
2. Why are hormones normally present in small amounts, even when they are not needed?
3. List three major actions (molecular effects) of hormones. Give an example of how one of these endocrine mechanisms affects the phenotype of an animal.
4. What is your definition of a hormone? Should a pheromone be considered a hormone?
5. How do hormonal systems function and why are hormones necessary?
6. Describe how hormones have selective action in specific tissues. Why is this important?
7. Why are there both intracellular and extracellular receptors?
8. Explain the role of negative feedback and positive feedback in regulating endocrine systems and give an example of each.
9. Using an example for each, describe the genomic and non-genomic effects of hormones, commenting on the time frame of each effect.

Section 1.2 Synthesis, release and metabolism of hormones

1. Using an example, describe the roles of pre- and prohormone sequences. Describe how the signal sequences direct newly synthesized protein for export from the cell.
2. Where in the cell are steroid hormones synthesized? What is the main enzyme system involved?
3. How are eicosanoids produced and what is their function?
4. Describe three major control pathways for the synthesis and release of hormones and give an example of each.
5. Explain how and why carrier proteins are important in modulating the function of lipophilic hormones.
6. Outline the pathways for the degradation and inactivation of peptide and steroid hormones.
7. Describe two ways that protein hormones are modified post-translationally to alter their function. Comment on whether this modification causes a short-term or long-term change to the protein.
8. Describe the potential roles of sulfation in regulating steroid hormone function.
9. Describe the mechanism for the synthesis of protein hormones and the process of movement and processing of newly synthesized protein in the cell.
10. Outline the steps that occur in the thyroid follicular cells and the colloid during the synthesis of thyroid hormones. Describe the effect of TSH on thyroid hormone synthesis.
11. Discuss factors that regulate hormone release and hormone metabolism and excretion.

Section 1.3 Receptors and hormone action

Receptors for protein hormones

1. Describe two different experiments to determine if a hormone acts via a cell surface receptor.
2. Discuss factors that regulate hormone signalling at the target tissue.

3. Describe the overall structure and mechanism of action of G protein-coupled receptors. How is signalling by these receptors terminated?
4. The LH receptor is a G-coupled receptor associated with $G\alpha_s$ type. What would be the effect of LH stimulation on cAMP levels in target cells? Why? What would happen if cells were treated with forskolin instead of LH?
5. A hormone X was shown to act via a G-coupled receptor associated with $G\alpha_q$ type. What would depleting cellular calcium do to the hormone action? Why?
6. Describe the genomic effects of cAMP/PKA and the role of CREB.
7. Describe experiments that could be performed to demonstrate that the cAMP/PKA system is involved in a particular hormonal response.
8. Describe two experiments to determine if a hormone acts through a G protein-coupled receptor and which G protein is involved in the hormonal response.
9. Why are EGF and insulin receptors called tyrosine kinase receptors? How do they work?
10. Describe methods to determine whether the Ca-dependent PKC pathway is involved in a hormonal response.
11. Outline the mechanism of action of cytokine receptors and serine kinase receptors.
12. Describe the role of MAP kinase in integrating the endocrine response to growth factors.
13. Explain the significance of the finding that the monomeric G protein Ras is found in an active form in many cancers.

Receptors for steroid hormones

1. What is a transcription factor?
2. Describe the function of the different subgroups of nuclear receptors, NR0 to NR6. What are orphan receptors?
3. Other than the hinge region, what are the three common structural elements of nuclear hormone receptors? What is the function of each of these elements?
4. What part of a receptor contains the zinc finger motif? What is its role in receptor function?
5. Outline the mechanisms of action of steroid hormones. What is the time frame for these different effects?
6. Describe the mechanism of how steroid hormone receptors activate hormone-responsive genes.
7. Briefly describe the 'gene reporter assay' technique. What do you use it for?
8. Describe two techniques that can be used to study DNA-binding proteins.
9. Discuss how histone modifying enzymes and non-histone high-mobility group (HMG) proteins affect the structure of nuclear chromatin and how this affects hormone action.
10. Describe the role of epigenetic changes in DNA.
11. Describe experiments to identify DNA sequences involved in hormonal regulation of gene expression.
12. Describe a mechanism of how different hormone responses can be integrated.
13. Describe a mechanism for the rapid non-genomic effects of steroids.

Section 1.4 Pituitary–hypothalamic integration of hormone action

1. Illustrate the position of the brain, hypothalamus and pituitary gland.
2. Describe the integration of hypothalamic and pituitary function (nervous/pituitary–hypothalamic portal system).
3. What is the nature of the hypothalamic releasing and release-inhibiting hormones (function and structure)?
4. What hormones are produced by the posterior pituitary gland (structure, synthesis and function)? What stimulates their release and how is this regulated?
5. What are the properties of the hormones produced by the anterior pituitary gland? How is their release regulated?
6. Describe the direct and indirect actions of somatotrophin.
7. Using a diagram, explain how the hypothalamic–pituitary–target gland axis is integrated.
8. Describe experiments used to demonstrate that the secretion of hormones by the anterior pituitary gland is under hypothalamic control. Describe the experimental steps, possible results and how they can be interpreted.
9. Describe the function and control of pulsatile release of pituitary hormones. What is one benefit to pulsatile hormone release? Experimentally, what can be done to account for the pulsatile nature of hormone release?
10. Describe how circadian rhythms are regulated via the transcription–translation feedback loop of the circadian clock. Why are they important?
11. Describe how endocrine systems can be regulated at the level of the synthesis and release of

hormones, transport and delivery of hormone to target tissue, receptor number and function or via downstream signalling events. Give an example of each type of regulation.

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2 Endocrine Methodologies

This chapter describes the different methodologies that are used in endocrinology. Different model systems to study endocrine systems from *in vitro* to *in vivo* are first described, along with the use of hormone agonists and antagonists and antibodies against hormones. Assay methods to measure concentrations of hormones are covered, including bioassays, competitive binding assays and chemical assays, along with methods to measure hormone–receptor binding and receptor numbers. Methods for producing both steroid and non-protein hormones and protein and peptide hormones are then described. Finally, methods for manipulating endocrine function, including administering exogenous hormones and use of antibodies to enhance or repress endogenous hormone action, are covered and the use of transgenic animals is described.

2.1 Methods for Studying Endocrine Function

Key concepts

- Many different model systems can be used to study endocrine systems. Use the most simple and well-defined system that will achieve your objectives, considering the advantages and disadvantages of each model.
- Whole-animal models measure the *in vivo* function and the net effects on the whole animal from the interplay among individual organs.
- Classical *in vivo* methods remove an organ, noting the effects, and then replace the organ or use an extract of the tissue to reverse the effects.
- Organs can be removed surgically or chemically, or inactivated by deafferentation, and tissues can be replaced at an ectopic site or an extract of the tissue can be injected.
- A perfused organ system is useful for determining the overall metabolic effects on a particular organ.
- Consider the strain and physiological state of the animal, dose of hormone and duration of treatment in whole-animal and organ perfusion studies.
- *In vitro* models are useful for studying the details of particular biochemical pathways involved in the endocrine effects on specific tissues or cell types.
- Cell culture systems use either primary cells or immortalized cell lines. Culture conditions are critical. Culture of cells to form organoids or organ-on-a-chip allows the modelling of cell–cell and organ–organ interactions.
- A number of hormone antagonists and agonists and metabolic inhibitors are available.
- Polyclonal antibodies can be generated against hormones and purified by affinity chromatography. Monoclonal antibodies against a single epitope are more specific. Aptamers are an attractive alternative to antibodies, being highly specific, stable and readily synthesized.
- Antibodies can be used to identify the site of hormone synthesis or target tissue or for immunomodulation of hormone action.
- Transgenic animals can be used to modify hormone responses, as model systems to study hormone function and disease modelling, or as bioreactors to produce a valuable protein product.

Introduction

The goal of 'applied endocrinology' is to use endocrine systems to improve or monitor animal performance, health and welfare. Endocrine systems regulate most aspects of the physiology of animals, and alterations in these systems can lead to not only improved performance but also various disease states. As such, endocrine systems are important targets for drug discovery, particularly in humans, and many drugs affect the activity of one or more endocrine systems. In order to use endocrine systems, first it is necessary to characterize an endocrine

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system and understand how it functions. In broad terms, this involves identifying the endocrine tissue and target organs and determining the physiological effects of hormones and their significance to the animal. The detailed information needed includes:

1. the source of the hormone (organ or cell type) and chemical structure of the hormone (for example, protein, steroid, amino acid or fatty acid derivative);
2. the details of the biosynthesis, storage, secretion and the stimulus for secretion of the hormone;
3. the transport of the hormone and the mechanism of recognition of hormone by the target cell, including the receptor type;
4. the mechanism of hormone action in the target cell, including the intracellular signalling pathways involved;
5. physiological effects of the hormone and their significance to the animal; and
6. the pathways of metabolism, inactivation and excretion of the hormone.

Experimental model systems

A number of *in vivo* and *in vitro* model systems can be used to study endocrine systems. Models range from the whole animal to isolated perfused organs, tissue slices, isolated cells in culture and subcellular fractions (Fig. 2.1). The model system of choice for a particular study depends on what information is needed and what level of organization is needed to achieve the objectives of the study. The *in vivo* model using the whole animal is the most physiologically relevant but the parameters of interest and the complexity of their integration are hard to control and account for. The clearance and excretion of test compounds have to be considered and there is biological variability from animal to animal. The *in vitro* models using either isolated organs or tissues, slices or pieces of organs, isolated cells or subcellular fractions are ideal to study a particular well-defined end point. It is easier to control for environmental and other interactions, but *in vitro* models are less physiologically relevant, so the results obtained with these models need to be placed back in context of the whole animal.

Generally, use the most simple and defined system that will achieve the objectives of the experiment. For example, the overall effects of an endocrine system on animal performance would need to be studied in a whole animal, while the mechanism of action of the hormone might best be studied in an isolated cell system.

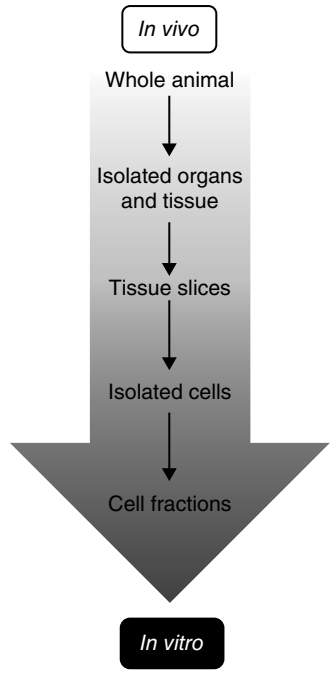


Fig. 2.1. Model systems for studying endocrine function.

Whole-animal model

Experiments using the whole animal have the advantage of measuring *in vivo* production and uptake of hormones by various organs. The true '*in vivo*' function and effect on the animal can be established in the whole-animal model. The net effects on the whole animal are determined and the interplay among individual organs can be examined. However, whole-animal experiments have the disadvantages of high cost for animals and reagents and high biological variability among different animals. Biological variability can be reduced somewhat by using highly inbred lines of animals with a similar genetic make-up.

The classical methods used to study endocrine tissues are *in vivo* whole-animal studies in which organs are removed and replaced. The approach is to remove the organ, note the effects on whole-animal function or on specific systems in the animal, and then replace the organ. Sham-operated animals, in which the surgery is performed up to the point that the organ would be removed but the animal is left intact, act as controls (Fig. 2.2). A potential problem with this approach is that the

organ might be vital or the effect observed can be indirect. A variation of this procedure is referred to as *ex vivo*, where an organ, cells or tissue are taken from an animal which may have undergone a specific treatment. The isolated tissues are then subjected to a treatment or procedure and may be returned to the living animal.

Organs can be removed surgically (= 'ectomy'), such as adrenalectomy or removal of the adrenals, which would result in lowered plasma adrenal steroids and catecholamines. Lower levels of glucocorticoids (e.g. cortisol) would result in decreased plasma glucose concentration and decreased response to stress. Lower levels of mineralocorticoids (e.g. aldosterone) would result in lower plasma sodium, due to decreased reabsorption of sodium and increased secretion of potassium in the kidney.

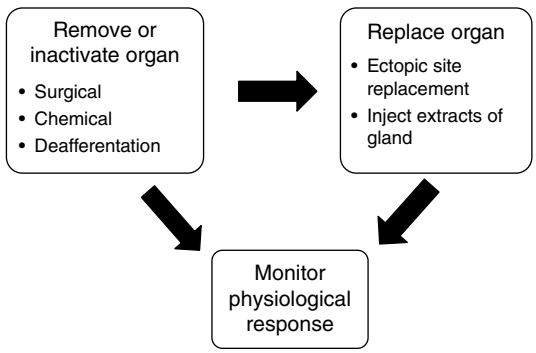


Fig. 2.2. Classical methods for studying hormone action in whole animals.

Decreased production of catecholamines would decrease the response to stress. In the case of paired organs, such as the adrenals and gonads, removal of one organ can cause the other to increase in size to compensate for the lost capacity. This can be from hypertrophy (increase in cell size) or hyperplasia (increase in cell number). Note that hyperplasia can be distinguished from hypertrophy by an increase in the DNA content of the tissue.

Another example of surgical removal of organs is hypophysectomy, or removal of the pituitary (hypophysis). Hypophysectomy demonstrates that the endocrine system is under pituitary control. For example, in the winter flounder, *Pseudopleuronectes americanus*, a hypophysectomized fish turns grey due to a lack of melanocyte-stimulating hormone (MSH) from the pituitary. In the absence of a physiological response, it is necessary to measure hormone levels in blood to make sure the surgery was successful.

When the hormone is removed from the circulation, the negative feedback that regulates the production of the hormone is removed. This can result in sustained high levels of trophic hormones; for example, levels of luteinizing hormone (LH) increase after ovariectomy, due to lack of negative feedback from gonadal steroids (Fig. 2.3; see also Fig. 1.58).

Organs can also be inactivated by treatment with chemicals or by removing the nervous stimulation of the tissue. Examples of chemical inactivation include treatment with alloxan, which destroys the β cells in the islets of Langerhans in the pancreas, which produce insulin. Cobalt chloride treatment destroys the α-cells, which secrete glucagon. This is

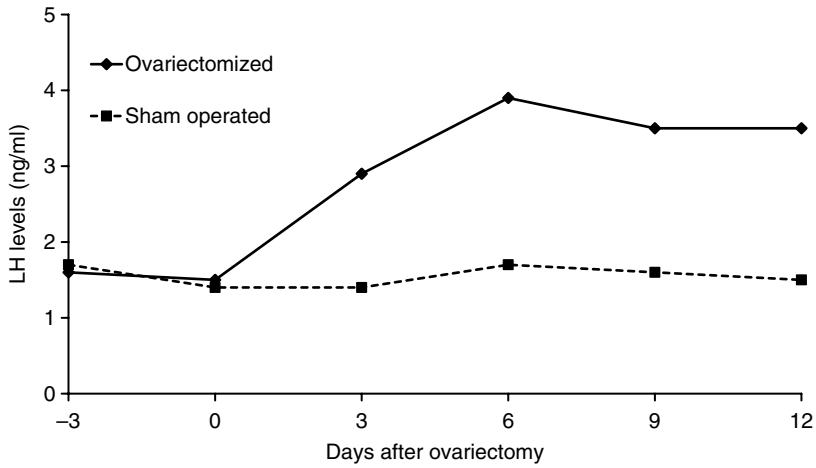


Fig. 2.3. Serum LH patterns after ovariectomy of chickens.

particularly useful when surgical removal is impossible, as in the islets cells, which are scattered throughout the pancreas. The roles of insulin and glucagon in glucose metabolism are summarized in Fig. 2.4.

Another example of chemical inactivation is radiotherapy, such as using radioactive iodine to destroy follicular cells in the thyroid gland that produce thyroid hormones. This results in decreased basal metabolic rate (the resting rate of calorie expenditure) and decreased heat production.

An example of removal of nervous stimulation to an organ, called deafferentation, is vagotomy or disruption of the vagus nerve to the gastrin-producing cells of the stomach. Normally the vagus nerve stimulates gastrin secretion in anticipation of food or distension of the stomach. Gastrin stimulates HCl and pepsinogen secretion into the stomach.

To confirm that the endocrine response is due to a particular tissue, the tissue can be placed back in the animal and a reversal of the effects from removal of the tissue should be seen. In these studies, it is necessary to control for rejection of the implanted tissue, and the use of highly inbred strains may minimize these problems. Tissues can be surgically transplanted to an ectopic site that is well supplied with blood and allows for delivery of the hormones, for example beneath the kidney capsule or within the orbit of the eye.

A variant of this process is parabiosis, in which the blood supplies of two animals are connected together (Fig. 2.5). One animal is treated and the effects in the other animal are noted, to demonstrate that blood-borne factors are causing the effect. For example, if the blood systems are connected between an incubating and egg-laying chicken, the egg-laying chicken will start to show signs of broodiness due to the effect of prolactin (see Section 6.1). This method has been used to compare the effects of new blood versus old blood (see 'For interest' box).

For interest

Parabiosis – Applications to Biology of Ageing: New blood versus old blood

Parabiosis was first described in 1864 by the French zoologist, Paul Bert. During the 1950s, Clive McCay of Cornell University showed that connecting the blood system of a young mouse to an old mouse rejuvenated the older animal. In a 2005 paper in *Nature*, Conboy *et al*. showed that young blood caused muscle stem cells to proliferate and regenerate muscle. This effect was subsequently linked to increased levels of GDF11 (part of transforming growth factor β superfamily) (Loffredo *et al*., 2013).

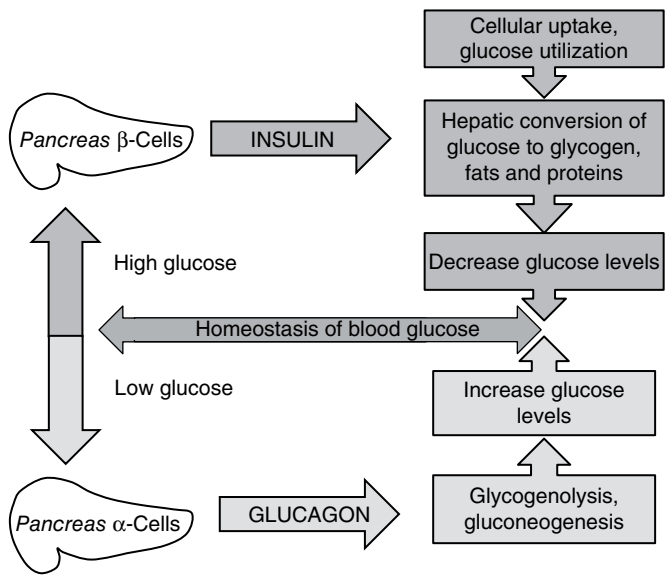


Fig. 2.4. Regulation of glucose metabolism.

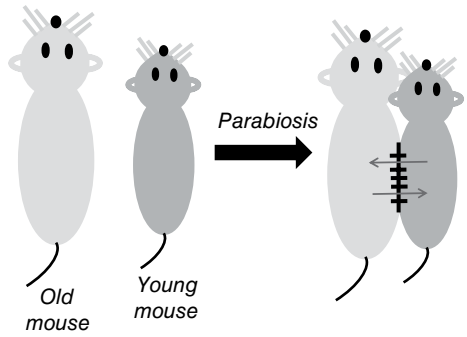


Fig. 2.5. Parabiosis connecting the blood systems of two animals together. One animal is treated and the effects in the other animal are noted, to demonstrate that blood-borne factors are causing the effect.

An extract of the gland can also be injected to check for the presence of the hormone by measuring the endocrine response. The active extract is then purified to obtain separate compounds with hormonal activity for use in hormone replacement therapy. For example, insulin-dependent diabetes type I resulting from the lack of insulin production by pancreatic β cells can be treated by daily injection of insulin. Obtaining the growth hormone necessary for the treatment of pituitary dwarfism used to involve purifying the hormone from the pituitary of cadavers at a high cost and high risk but, with the development of recombinant DNA technology, recombinant hormones are available (see Section 2.3). With hormone replacement therapy, the risk of contamination, source and purity of the hormone are important.

In whole-animal studies where the effects of various hormones are studied *in vivo*, the following issues should be addressed.

1. What animal would be used? Is a smaller but still relevant animal model available (e.g. use sheep instead of cattle)? Should inbred strains or specific genetic crosses be used?
2. What dose of hormone would be used? Is this a physiological or pharmacological level?
3. What is the appropriate physiological state of the animal (e.g. age, maturity, reproductive status)?
4. What is the appropriate route of administration (in the feed, by injection–implant or multiple injections; see Section 2.4)?
5. What are the potential interactions or side effects of the treatment?
6. What dependent variable(s) will be measured?
7. What are the advantages and disadvantages of this model?

Isolated organs or tissues

An isolated perfused organ system is useful for determining the overall metabolic effects on an organ, for example the effects of insulin/glucagon on glucose metabolism. It could also be used to study hormone production by an organ in response to blood-borne signals, such as trophic hormones or levels of metabolites. The blood vessels supplying and draining the organ or tissue are cannulated and the vascular system is perfused with a modified blood cocktail that is continuously oxygenated and recirculated (Fig. 2.6). The system has the advantages of lack of effects from endogenous hormones and neural influences, as it isolates the effects of other tissues in the animal. The system is also easily manipulated and sampled. The disadvantages of the system are that surgical skill is required and, since it is not *in vivo*, it can sometimes be difficult to maintain organ viability.

Another version of this is the use of isolated organs or tissues *in situ*. In this model, catheters are placed in the blood vessels supplying and draining the organ or tissue and the organ is left in the circulation and not removed from the animal. Blood samples can be taken from the arterial and venous catheters to determine what effects the organ has on levels of hormones or metabolites. Test compounds can be supplied to the organ via the arterial catheter and the effects on the organ noted. An example of this would be the *in vivo* catheterization of the vessels supplying the mammary gland in dairy cattle (Fig. 2.7). Separate catheters are placed in the external pudendal arteries supplying the two sides of the mammary gland. This split-udder design allows one side of the mammary gland to be used for a treatment, while the other side is used as a control (see Section 4.1).

With the organ perfusion/infusion model, the following factors should be considered.

1. What is the appropriate animal (species, strain, etc.) to study the effect?
2. How will the hormone be given in the perfusion/infusion?
3. What dose will be used and how long will the treatment be carried out?
4. What is the appropriate physiological state of the animal (age, maturity, reproductive status, etc.)?
5. What dependent variable(s) will be measured?
6. What are the advantages and disadvantages of this experimental approach?

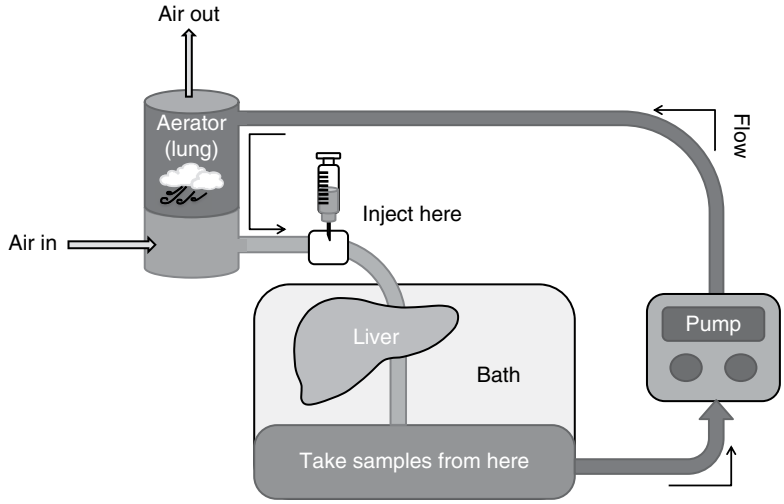


Fig. 2.6. Organ perfusion system.

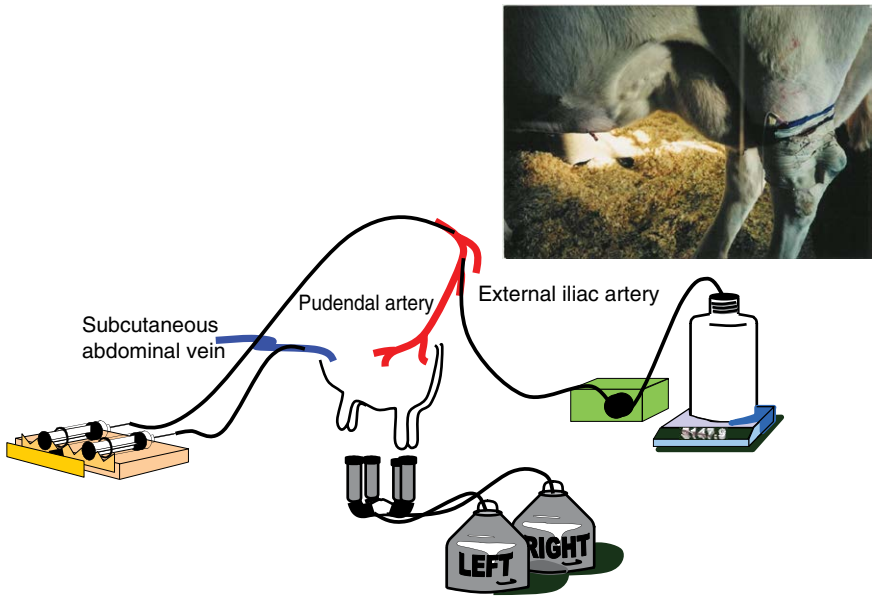


Fig. 2.7. *In situ* perfusion of mammary glands. The external pudendal arteries supplying the two sides of the mammary gland allow a split-udder design of experiments (Maas *et al.*, 1995). (Used with permission, John Cant, University of Guelph.)

In vitro *models*

In vitro models are useful for studying the details of particular biochemical pathways involved in the endocrine effects on specific tissues or cell types but they suffer from several limitations. First, the tissue being studied tends to be in a catabolic state when studied *in vitro*, so the results can be somewhat qualitative rather than quantitative. Secondly, the results can be dramatically affected by the composition of the incubation medium, since the presence of particular hormones or metabolites can alter the activity of metabolic pathways. Thirdly, the concentration of hormones used may be near-pharmacological rather than physiological and thus the

results obtained *in vitro* can differ dramatically from the results *in vivo*. However, because the tissues have no connection with the endocrine and nervous systems, they are not subject to multiple factors present in blood. These defined *in vitro* systems are thus ideal for the study of hormone action on a specific target but not good for long-term studies of the action of hormones.

The next lower level of organization from isolated organs or tissues is the use of thin tissue slices that are incubated in an appropriate medium containing necessary metabolites and oxygen for the cells to remain viable. This has the advantage of being technically easier than perfusion, not requiring blood cells, and allowing for the study of different areas of the same organ. However, the slices must be very thin, so that oxygen can penetrate through to the cells on the interior of the slice, and there are many damaged cells on the surface of the slice. They are also widely used for immunocytochemistry (see below) and cell morphology studies.

Cells from different areas of a tissue slice can also be captured using laser capture microdissection and be used to measure the levels of metabolites or gene expression in specific cells. This technique uses a laser coupled to a microscope; the laser focuses onto the tissue on the microscope slide and is used to cut out specific cells from the tissue sample. For example, cells surrounding the portal vein and bile duct (periportal zone I) can be captured and compared with cells surrounding the hepatic vein (centrilobular zone III) in liver slices (Fig. 2.8).

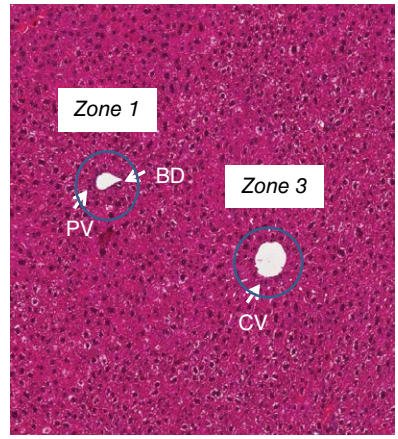


Fig. 2.8. Section of mouse liver, magnification ×200 with H&E staining and zone 1 and 3 outlined. CV, central vein (hepatic vein); PV, portal vein; BD, bile duct.

Cell culture systems

Cell culture is the process of growing cells under controlled conditions outside their natural environment. Isolated primary cells can be prepared by treating a living tissue with collagenase to break down the connective tissue and release the cells. Various types of cells can also be isolated, so that the contribution of different cell types to the overall metabolism of a tissue can be studied. This can be accomplished using gradients of Percoll™ or similar non-toxic reagents that separate cells based on their density. The viability of the cells is determined by their exclusion of the dye, trypan blue. The cells are then maintained under controlled conditions that are specific for each cell type. The growth medium supplies the essential nutrients (amino acids, carbohydrates, lipids, vitamins and minerals), growth factors and hormones, and the appropriate balance of CO_2 and O_2, pH and temperature is maintained.

Isolated cells have the advantage of being a more defined system, which can be used to study the effect of hormones on the activity of particular genes and other molecular responses at the cellular level. This includes the effects of cellular growth factors or studying the control of gene expression (hormone-responsive elements, etc.) (see Section 1.3). Various inhibitors can be used to determine the mechanism of action of hormones within the cell. This system uses fewer animals and many treatments can be performed from one preparation of cells, with control and treatments using the same cells, which reduces biological variability.

However, cell culture is not an *in vivo* system and the culture conditions are critical (for example the presence of serum, type of coating on culture dishes, plating density) as artefacts can be produced inadvertently if the conditions are not controlled carefully. Cell viability and receptor/enzyme functions can also change over the time that the cells are in culture, as the cells tend to dedifferentiate. Because specialized cells are differentiated and do not divide, they have a limited culture time.

Cells can be grown on the surface of a culture dish or an artificial substrate (adherent or monolayer culture) or a three-dimensional support matrix (for 3D culture) or be free floating in culture medium (suspension culture). Some cells need to be grown with a layer of feeder cells, which are growth arrested but viable and bioactive cells (typically

fibroblasts) that produce a variety of soluble or membrane-bound growth factors that the specialized cells require for optimum growth.

Explant culture is a technique to culture cells from a piece of tissue or organ. Small pieces of tissue are surgically removed from the animal and are aseptically placed on a tissue culture plate with a coated surface and allowed to attach to the surface in the presence of a rich culture medium. The explants are then cultured in standard tissue culture conditions, and after 15–30 days progenitor cells migrate out of the tissue onto the surface of the dish.

A number of tissue-specific cell culture systems have been developed to simulate the conditions that cells normally experience within a particular tissue in order to better model the *in vivo* conditions within an organ. The microenvironments in which cells grow include important mechanical and chemical signals that affect cellular function. A variety of cell types can also be placed in proximity to each other and thereby create multi-cell-type tissue constructs as well as tissue–tissue boundaries. Recent progress in microtechnology has enabled manipulation of cellular environments and the development of novel *in vitro* organ systems, termed organ-on-a-chip systems. By mimicking the cellular environment of *in vivo* tissues, these organ-on-a-chip systems can reproduce specific organ functions better than conventional *in vitro* model systems and thus make the system more physiologically relevant. These systems can also be linked together into multi-organ body-on-a-chip systems that reproduce the dynamics of the whole-body response (Fig. 2.9).

To model the lung, a two-layer device has been developed to allow controlled mechanical stretching of the endothelial–epithelial bilayer, which simulates the mechanical stresses that are present in the lung during breathing. When mechanical stretching was applied to the bilayer system, organ-level responses to bacteria, adhesion of neutrophils and pathogen phagocytosis were observed.

The cells making up the functional unit of the liver, the acinus, express a different set of proteins depending on their location within the unit. Cells in the periportal zone I are nearest to the entering blood supply and receive the most oxygenated blood, while cells in the centrilobular zone III have the poorest oxygenation (see Fig. 2.8). Simulating this oxygen gradient replicated the expression of different cytochrome P450 enzymes that is found *in vivo*. Co-culture of primary hepatocytes and stromal cells improved various liver-specific functions and the endothelial–hepatocyte interface of the liver induced hepatocytes to organize into bile canaliculi.

The GI tract is under complex mechanical movement, including segmental contraction, peristaltic waves and movement of villi. It is also occupied by a large number of microbes (the microbiome; see Section 3.11) which coexist with the intestinal epithelial cells. A novel hydrogel microfabrication method has been used to create a collagen scaffold to culture cells into a three-dimensional (3D) villi shape.

The blood–brain barrier can be replicated using transmembrane culture plates with brain microvascular endothelial cells cultivated on the top side of the membrane and astrocytes and pericytes cultivated on the bottom side. A microfluidic system controls flow between the cells and integrated electrodes measure electrical activity. Two neuronal populations can also be interconnected through microchannel networks and the electrical activity of the cells recorded.

To model heart function, the force generated by sheets or films of cardiomyocytes can be measured by growing the cells on polydimethylsiloxane (PDMS) cantilevers, PDMS films mounted on posts and PDMS films attached at one end. A film of cardiomyocytes has also been incorporated onto a diaphragm: when the cardiomyocytes contracted, the diaphragm deformed and produced a change in pressure in the chamber.

To replicate muscle, 3D culture systems are used where myoblasts are grown on synthetic scaffolds and fuse to form parallel arrays of myotubes capable of contraction. They organize along the lines of a physical strain provided by cellular contraction against fixed posts. Attachment of these posts to a force transducer allows for the calculation of the force generated by the cells.

Several microfabricated organ-on-a-chip systems can be integrated to simulate the whole-body responses in a body-on-a-chip device, where tissue chambers are connected by a set of microfluidic channels that are similar in diameter to microvascular capillaries (see Fig. 2.9). The flow rate can be controlled so that the residence time of the fluid in each chamber matches the residence time of corresponding organs in the body. Body-on-a-chip cell culture systems can simulate tissue–tissue interactions in a more physiologically realistic manner.

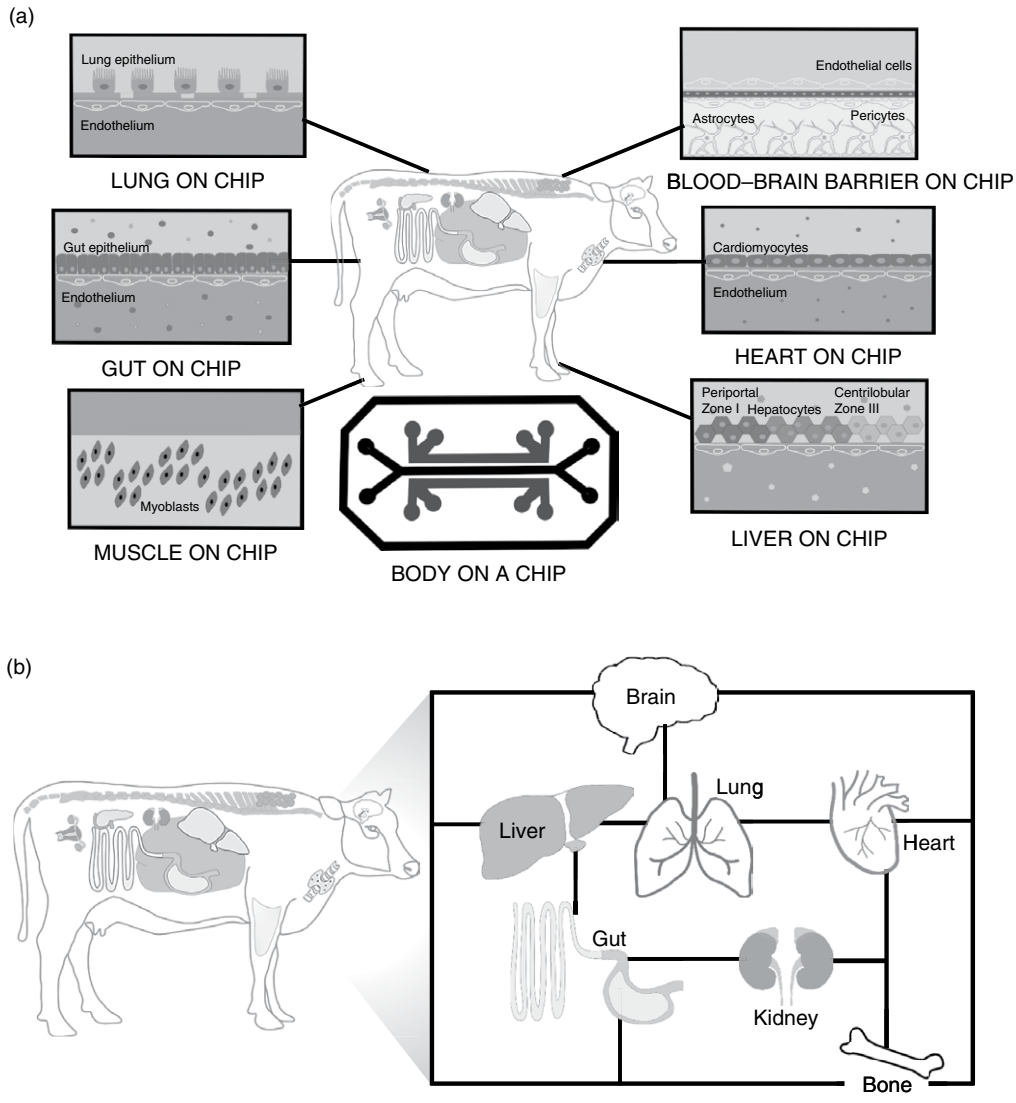


Fig. 2.9. (a) Organ-on-a-chip systems. **(b)** Integration of different organ chips to form body-on-a-chip systems.

These systems can be a valuable tool for studying multi-organ interactions, similar to physiologically based pharmacokinetic (PBPK) modelling, which mathematically segregates the body into organ compartments connected via hypothetical blood flows. For example, a three-way interaction between the hepatocytes (liver), adipose tissue (fat) and endothelial cells (blood vessel) on glucose metabolism was studied. By controlling the glucose level in the perfused medium, normal and hyperglycaemia were mimicked, and the response of each tissue model to insulin was measured. For more information, see the review by Sung *et al*. (2013).

An alternative to primary cell culture is to use established immortalized cell lines that have been derived from carcinomas from a particular tissue and species of animal. This eliminates the need to collect tissue from animals, and many of these cell

lines can be obtained commercially, for example from the American Type Culture Collection (ATCC), the European Collection of Cell Cultures (ECACC) and the German Collection of Microorganisms and Cell Cultures (DSMZ). Other cell lines have been experimentally immortalized by transforming primary cells with tumour-inducing virus or chemicals. An example of this is the MAC-T3 cell line derived from mammary alveolar cells transfected with large T antigen. Immortalized cells produce a stable long-term system, since they keep on dividing and produce a large amount of identical cells that all originated from one clonal line. The cells are easy to grow and manipulate; for example, they can be readily transfected with gene constructs and are particularly useful for studying gene expression and producing recombinant proteins (see Section 2.3) or as encapsulated cells that can be implanted in an animal (see Section 2.4). Since they are modified cells, they do not act exactly like primary cells, so results from metabolic studies obtained using these cells should be interpreted with caution.

For interest

The Lacks Legacy – HeLa cells

HeLa cells are one of the earliest human cell lines and the line was derived from a cervical cancer from Henrietta Lacks. In 1951, a biopsy of Henrietta Lacks' tumour was collected without her knowledge or consent and used to establish the HeLa cell line. In 1971 several journals named Henrietta Lacks as the HeLa source and a few years later the Lacks family members learnt about HeLa cells. Scientists later collected their blood to map HeLa genes. In 1996, the Lacks family was honoured at the first annual HeLa Cancer Control Symposium, organized by a former student of the scientist who isolated HeLa cells. In 2013, the HeLa genome was published without knowledge of the family, which later endorsed restricted access to HeLa genome data.

The static environment of a tissue culture system results in the accumulation of metabolites and secreted factors, leading to possible artificial paracrine and autocrine effects. There is also normally a pulsatile presentation of many hormones to target cells occurring *in vivo*, as well as a continuous blood flow, which brings fresh nutrients and removes hormones and other factors secreted by the cells. An alternative to static tissue culture is the perifusion system for cell culture, which can offer the advantage of cell culture with a more physiological 'twist'. In the perifusion system (Fig. 2.10), media or media supplemented with hormone are continuously supplied to the cell culture at a desired rate using a series of pumps regulated by a controller. Thus the cells are exposed to fresh media, and hormones can be delivered either continuously or in a pulsatile manner.

With the cell culture model system, the following points should be considered.

1. What is the appropriate cell type? Is a primary cell culture or immortalized cell line appropriate?
2. What are the appropriate conditions for cell culture? Can a defined medium be used or is serum required? What culture surface is required (e.g. collagen-coated plates or plastic or 3D scaffold)? Is a feeder cell line required in co-culture? Is a perifusion system appropriate?
3. What is the appropriate duration of treatment? Are cell viability and response to hormones maintained (e.g. receptors maintained)?
4. What are the appropriate end-point measurements (e.g. specific proteins produced, cell growth, etc.)?
5. What is the appropriate dose of hormone for culture conditions (physiological versus pharmacological)?
6. What are the advantages and disadvantages of this experimental model?

Various subcellular fractions can be prepared by homogenizing cells or tissue in buffer and then centrifuging the homogenate at different speeds (differential centrifugation) to obtain the different fractions (nuclei, mitochondria, membrane). These fractions can be used to study specific processes, for example membrane transport using membrane vesicles or enzyme activities, or structure/function relationships of molecules involved in endocrine responses. The advantages of subcellular fractions are their usefulness for detailed biochemical studies and that they represent a well-defined system. However, this system can produce artefacts, particularly as high levels of compounds are used, so the results may not reflect what is found in an *in vivo* system.

Use of agonists, antagonists and inhibitors

Agonists are substances that bind to a hormone receptor and are able to mimic the effect of the

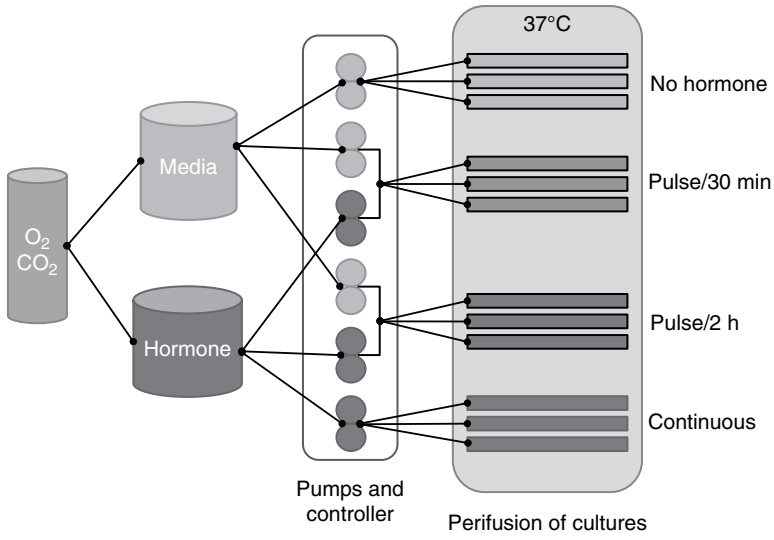


Fig. 2.10. Perifusion system for tissue culture.

hormone, while antagonists are substances that block or inhibit the effect of hormone at the receptor. Inhibitors block the action of hormones but act downstream of receptors. They can be specific and affect only one pathway, or non-specific and alter general cell metabolism. These include inhibitors of the synthesis of DNA, RNA and protein, metabolic inhibitors and compounds that affect membrane transport. These compounds are useful for determining the mechanism of hormone action, particularly in studies using cell culture or subcellular fractions.

A number of hormone antagonists and agonists are available. These are of great pharmacological value, and Sir James W. Black shared the Nobel Prize in Physiology or Medicine 1988 for his discovery of propranolol and cimetidine, which inhibit the β-adrenergic receptor and the H2 histamine receptor, respectively. Propranolol and its derivatives are β-blockers that are used in the treatment of heart conditions and elevated blood pressure. Cyproterone acetate antagonizes testosterone binding at androgen receptors and can be used to reduce aggression and to treat prostatic hypertrophy. Dexamethasone is a synthetic glucocorticoid agonist that is widely used; and buserelin is a GnRH agonist that is ten times more potent than GnRH (see also Table 5.1). Chlorpromazine and ergot alkaloids (e.g. ergocryptine) are dopamine receptor antagonists. Spironolactone is an aldosterone receptor antagonist and saralisin is an angiotensin II receptor antagonist. Thiouracil inhibits the uptake of iodide and synthesis of thyroid hormone in the thyroid gland.

Several compounds can be used to study GPCR systems, including cAMP analogues, phosphodiesterase inhibitors, inhibitors of adenylate cyclase (SQ 22,536) and MAPK (PD 98,059) and activators of PKA and PKC (see Section 1.3, Table 1.2)

Various inhibitors are available for the study of endocrine systems (Table 2.1).

Inhibitors of RNA synthesis include actinomycin D, which intercalates with the double helix of DNA to prevent transcription, and rifampicin, which inhibits transcription in prokaryotes. Inhibitors of protein synthesis include cycloheximide and puromycin. Cycloheximide inhibits the peptidyl transferase activity of the 60S ribosomal subunit. Puromycin resembles the aminoacyl terminus of aminoacyl-tRNA and joins to the carboxyl group of the growing peptide chain, causing it to dissociate from the ribosome and cause premature termination of the amino acid chain.

Inhibitors of microtubules and microfilaments include colchicine and cytochalasin B. Colchicine is a plant alkaloid that inhibits microtubule assembly by binding with tubulin. It inhibits insulin secretion, suggesting that microtubules may function in the secretion of this hormone. Cytochalasin B is a fungal metabolite that specifically interferes with

Table 2.1. Inhibitors useful for the study of endocrine systems

Inhibitor type	Inhibitor	Inhibitor effects
Transcription and translation	Actinomycin D	RNA synthesis
	Rifampicin	
	Cycloheximide	Protein synthesis
	Puromycin	
Microtubules and microfilaments	Colchicine	Microtubule assembly
	Cytochalasin B	Microfilament function
Metabolic inhibitors	Iodoacetic acid	Glucose utilization
	2-Deoxyglucose	
	Dinitrophenol	ATP formation
	Oligomycin	
Cytotoxic agents	Alloxan	Insulin production
	Cobalt chloride	Glucagon production
	Ionophores A23187 and valinomycin	Intracellular ion concentrations
Ion transport	Verapamil	Calcium channel antagonist
	Liposomes	

microfilament function without affecting microtubule integrity. It inhibits secretion of several hormones, suggesting that filamentous organelles may be involved in the secretory process of these hormones.

Metabolic inhibitors include iodoacetic acid, which blocks glycolysis, and 2-deoxyglucose, which inhibits glucose uptake and utilization within cells. Inhibitors that affect ATP formation include dinitrophenol, which uncouples oxidative phosphorylation, and oligomycin, which prevents mitochondrial phosphorylation of ADP to ATP. The cytotoxic agent alloxan destroys the β cells of the pancreas that secrete insulin, while cobalt chloride destroys the α-cells of the pancreas that secrete glucagon.

Ion transport can be affected by treatment with ionophores, which span biological membranes to carry ions into cells. The ionophore A23187 is used to increase intracellular Ca^{2+} (see Section 1.3), and valinomycin has a high specificity for K^+ transport. Verapamil is a calcium channel antagonist. Liposomes can be used as transport vehicles that fuse with the cell membrane and transfer their contents into the cytoplasm. Liposomes that incorporate specific proteins can be potentially targeted to different cells (see Section 2.4). This provides a novel method of studying the mechanisms of stimulus–secretion coupling, particularly as it relates to the release of a chemical messenger.

Use of antibodies

Antibodies (Ab) are produced by animals as a defence against foreign compounds, called antigens (Ag). Antibodies are widely used in endocrine studies and can be prepared by injecting hormones or receptors obtained from one species into another species.

Immune response

There are two types of specific immune responses: the cell-mediated response and the humoral response (see Section 6.3). The cell-mediated response is involved in the destruction of 'self' cells gone wrong, such as infected or cancerous cells, and is carried out by cytotoxic T lymphocytes. The humoral response is the generation of soluble antibodies against foreign antigens.

The humoral response is due to B lymphocytes, which are pre-committed to respond to a limited number of antigens. The initial exposure to an antigen produces the primary immune response, in which specific B cells that respond to a particular antigen divide and differentiate (Figs 2.11 and 2.12). The majority of the responding B cells become memory cells and others become antibody-producing plasma cells. Thus, there is only a small production of antibodies from the primary response. The secondary immune response occurs upon subsequent exposure to the same antigen. This results in a rapid proliferation and differentiation of memory cells into antibody-producing plasma cells and high antibody titres (a titre is the highest dilution of serum that gives measurable antibody response).

A large number of different antibody-producing cells are generated and each of these produces a unique antibody, which is directed against a particular antigenic site called an epitope on the surface of the

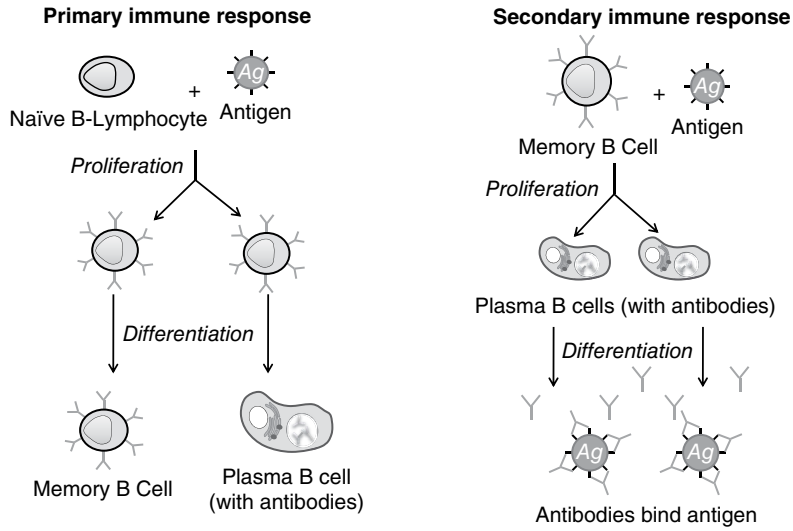


Fig. 2.11. The humoral response to foreign antigens.

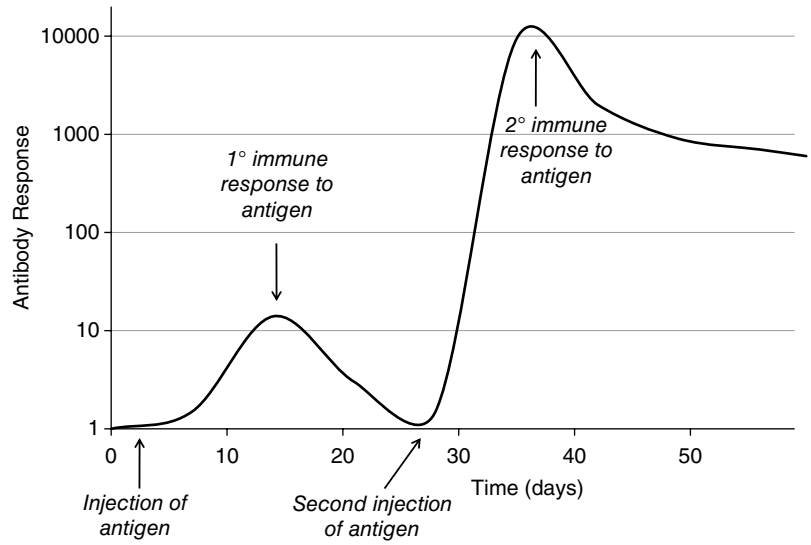


Fig. 2.12. Primary and secondary responses to antigen exposure.

antigen. Thus, the immunization process generates a mixture of antibodies, called a polyclonal antibody preparation (Fig. 2.13).

To elicit an antibody response, the substance (Ag) must be recognized as foreign (non-self) and be large (for example, proteins) and complex in structure (not a homopolymer such as polylysine). A hapten is a low-molecular-weight compound that can react with an antibody but is unable to induce antibody formation by itself. It must be attached to a larger antigenic molecule, such as keyhole limpet haemocyanin (KLH) that will readily be recognized as foreign to be antigenic. Protein hormones are usually antigenic, since their structures differ between species. Steroid hormones have a low molecular weight and their structure is the same in different species, so they need to be linked to a large protein to make them antigenic.

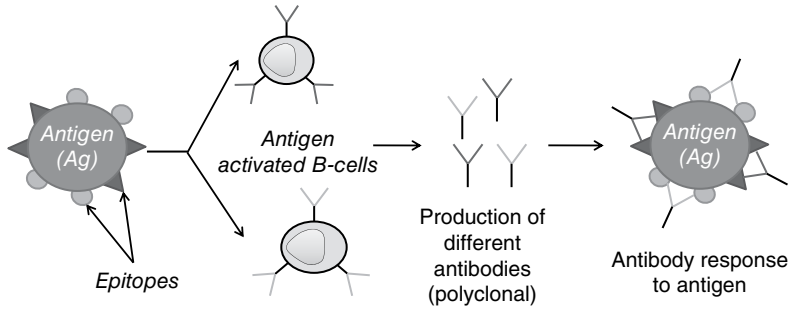


Fig. 2.13. Basis for polyclonal antibody production.

Another approach for generating antibodies against proteins is the use of DNA and RNA vaccines. A DNA vaccine involves the direct injection of an expression plasmid encoding a foreign protein into muscle, which results in uptake of DNA and expression of the foreign protein in cells and subsequent stimulation of the immune system. An RNA vaccine involves injecting RNA encoding a foreign protein; their application has until recently been restricted by their instability and the inefficient *in vivo* delivery of mRNA. Recent technological advances have now largely overcome these issues. Several modifications, including the 5′ cap, optimizing the poly A sequence and replacing rare codons with frequently used synonymous codons, make the mRNA more stable and highly translatable. Efficient *in vivo* delivery can be achieved by formulating mRNA into carrier molecules such as cationic lipids, polymers and nanoparticles, allowing rapid uptake and expression in the cytoplasm.

The advantages of these types of vaccines are that plasmids and RNA are easily constructed and the sequence can be readily manipulated to produce antibody responses against different protein variants. More than one protein (Ag) can also be expressed. They are more temperature stable than traditional vaccines, depending on the formulation, and they produce both cell-mediated and humoral responses, so they are good for chronic viral infections. The disadvantage is that they only express protein antigens. For more information on mRNA vaccines, see the review by Pardi *et al.* (2018) and for DNA vaccines see the review by Kutzler and Weiner (2008).

Normally an adjuvant is given with the antigen to enhance the immune response. A classic adjuvant is Freund's complete adjuvant, which is an emulsion of water in oil containing killed bacteria. It causes a slow, continuous release of antigen and local irritation, which stimulates macrophage activity but can cause discomfort for the animals. The immune response can sometimes also be increased by producing a polymer that contains many copies of the antigen within the same molecule.

For interest

Find examples of DNA and RNA vaccines that are currently licensed for use in animals.

Detection and purification of antibodies

The presence of an antibody against a specific antigen can be detected using immunodiffusion (Ouchterlony) plates (Fig. 2.14). Serum containing antibodies (antiserum) is placed in one well of an agar plate and the antigen is placed in an adjacent well. The two solutions diffuse through the agar and meet. The presence of an immunoprecipitate between the two wells indicates that an antibody is present that recognizes the antigen.

The antibodies can be purified from serum by affinity chromatography (Fig. 2.15). The Protein A column will bind the IgG fraction of serum which will contain a variety of antibodies. To purify specific antibodies that only recognize the antigen of interest, the antigen is linked to a support such as Sepharose to make an affinity column. The serum is applied to the affinity column and the antibodies bind to the column. The column is then washed with buffer to remove the unbound substances and the antibodies are then eluted from the column using a low pH buffer.

Monoclonal and recombinant antibodies

Antibodies produced *in vivo* are polyclonal since they are produced from more than one B cell clone,

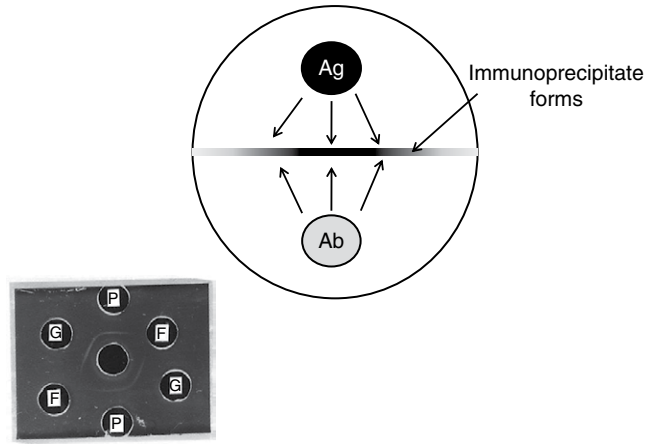


Fig. 2.14. Ouchterlony immunodiffusion experiment. The insert shows an experiment with Ab in the centre well and various Ag in the outer wells. Note that Ags 'G' and 'P' react with the Ab, while Ag 'F' does not react.

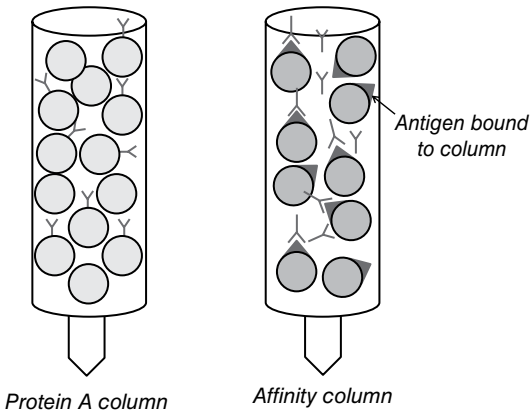


Fig. 2.15. Purification of antibodies using a Protein A column or an affinity column with the antigen bound to the column matrix.

with each B cell clone recognizing a specific epitope on the large antigen molecule (see Fig. 2.13). Serum also contains antibodies against other molecules and this may cause a non-specific immune response. Polyclonal Ab are relatively inexpensive and quick to produce. They can have a higher overall antibody affinity against the antigen and a greater sensitivity for detecting proteins in low quantities, due to the presence of multiple antibodies binding to multiple epitopes. The disadvantage of the polyclonal Ab is the variability between different batches produced in different animals at different times. There is also a higher potential cross-reactivity, due to recognition of multiple epitopes.

It is possible to produce antibodies from one clone (a monoclonal Ab) to increase specificity, since a monoclonal antibody recognizes only one particular epitope (Fig. 2.16). Once a mouse has been immunized with the antigen, the spleen is removed and the cells are isolated. The antibody-producing cells are then fused with a myeloma cell line to produce immortalized hybridoma cells. A selection process is used to isolate the hybridoma cells from the unfused myeloma cells and spleen cells. The myeloma cell line is missing the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) from the purine salvage pathway. The cells are grown in hypoxanthine–aminopterin–thymidine (HAT) medium containing hypoxanthine, thymidine and the synthetic folic acid analogue aminopterin, which blocks the thymidylate synthetase enzyme and forces the cells to use the purine salvage pathway. The unfused myeloma cells cannot survive under these conditions and unfused spleen cells cannot last more than a few days in culture. Thus, only the hybridoma cells that express the HGPRT from the spleen cells survive in the HAT medium. The individual hybridoma cell clones are then purified to produce cell lines that produce one particular monoclonal antibody. The cell line that produces the monoclonal antibody with the desired specificity is then used to produce large amounts of antibody.

The advantages of monoclonal Ab are that large quantities of identical Ab can be produced with no batch-to-batch variability and no animals are required once the hybridoma cell lines are established.

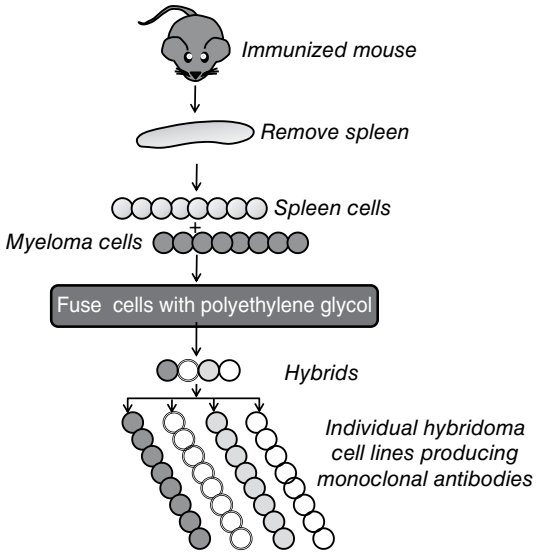


Fig. 2.16. Production of monoclonal antibodies.

They are highly specific to a single epitope and thus have a lower probability of cross-reactivity than polyclonal Ab. However, they are more expensive and require more time to produce. Small changes in epitope structure can also make the antibody unable to detect a target protein and they are more susceptible to binding changes when labelled. For more information, see Goding (1996).

Recombinant antibodies (rAbs) are monoclonal antibodies generated *in vitro* using synthetic genes. This involves creating an antibody gene library that encodes the variable regions of different antibodies, amplifying and cloning the genes into an appropriate phage expression vector (library display), and selection of individual antibodies from the library that bind to a specific antigen. The selected antibody genes are then expressed in a host (bacteria, yeast, or mammalian cell lines) to produce adequate amounts of functional antibody. With this technology, one can manipulate antibody genes to generate new antibodies and antibody fragments. rAbs also have the advantage that they do not need hybridomas or use animals in the production process.

Bispecific antibodies (bsAbs) combine the specificities of two antibodies for different antigens in one molecule. They were initially produced by coupling two monoclonal Abs together but are now produced using recombinant methods to fuse genes encoding two binding domains. They can be used to place targets into close proximity to support protein complex formation or cell–cell interaction and are used in cancer treatment to interfere simultaneously with two signalling pathways. For more information, see the review by Kontermann and Brinkmann (2015).

An alternative to antibodies is aptamers, which are single-stranded DNA or RNA molecules that fold into selective, high-affinity binding pockets for a target molecule. The targets range from small molecule drugs and toxins to proteins and even whole cells. Aptamers that bind to a specific target molecule are isolated from a randomized library of DNA or RNA sequences through a process called SELEX (systematic evolution of ligands by exponential enrichment), where sequences that bind to the target molecule are subjected to iterative selection rounds to increase the number of high-affinity aptamers until they eventually dominate in the library (Fig. 2.17). A selection cycle typically starts with a DNA or RNA library containing a random region of 20–60 nucleotides flanked by fixed primer regions at the 5′ and 3′ ends. The iterative selection cycles consist of binding to the target molecule, partitioning and recovery of the binding sequences, and re-amplification of the sequences by PCR using the fixed primer ends. This enriches the amount of specific aptamers that bind to the target with high affinity and these are then subjected to several more rounds of selection. The final aptamers are then isolated from the library and sequenced.

Aptamers have several advantages over antibodies. High-purity aptamers of known sequence can be chemically synthesized at a low cost with no batch-to-batch variation. They can be easily modified by conjugation with different dyes and labels without affecting their affinities. They can bind with high affinity; they are more stable to pH and temperature changes, have a longer shelf life than antibodies and can be reversibly denatured without loss of specificity. This makes them highly attractive in the development of low-cost, robust diagnostics and biosensors (aptasensors). For more information on aptamers, see the review by Dunn *et al*. (2017).

Use of antibodies to identify the site of hormone synthesis or target tissue

Antibodies against hormones can be tagged with a fluorescent enzyme or electron-dense probe and used to identify hormones present in hormone-producing or target cells. Labelled antibodies will

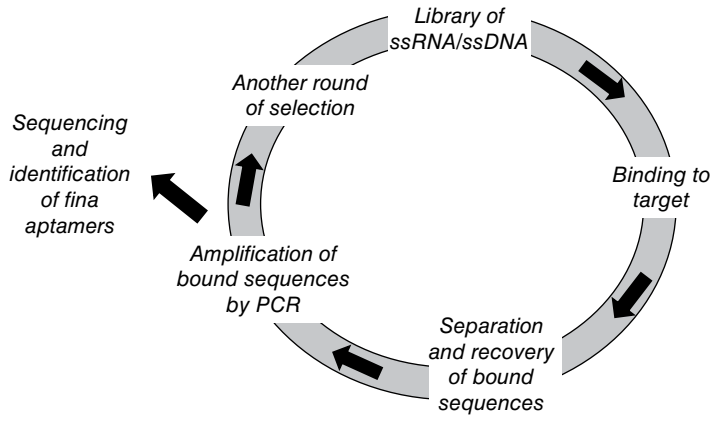


Fig. 2.17. SELEX procedure for isolation of aptamers.

accumulate in cells containing the hormone, which is recognized by that antibody. For example, in immunocytochemistry, labelled antibody is applied to histological sections of tissue to check for Ab binding using fluorescent-light microscopy or electron-dense probe via electron microscopy. In immunoenzyme histochemistry, an enzyme that gives colour reaction is coupled to the antibody to increase the sensitivity of detection.

Labelled hormone can also be used to localize areas of hormone binding. Radiolabelled hormone is injected, the tissue is sampled and fixed and the label is detected by autoradiography. This involves exposing the tissue to X-ray film and allowing the decay of radioisotope to produce spots on the exposed film. A related technique, *in situ* hybridization, is used to detect the presence of mRNA for a hormone or receptor in a particular cell type. In this case, the tissue section is exposed to a radiolabelled DNA probe for the hormone or receptor, and the binding of the probe to the tissue section is determined by autoradiography.

Antibodies can also be used to neutralize the effect of a hormone or to modulate hormone action, as described in Section 2.4. This can be done by passive immunization, in which the purified antibody is infused, or by active immunization, in which the animal produces its own antibodies against the hormone antigen. Antibodies can also be used for a variety of assays (see Section 2.2). Information on available antibodies against a particular target that are validated for particular applications is available on the antibodypedia reagent portal (http://www.antibodypedia.com).

2.2 Measurement of Hormones and Receptors

Key concepts

- Assays must show a dose–response relationship, have sufficient sensitivity, accuracy and precision, and be independent of sample matrix effects.
- Assays for hormones include bioassays, immunoassays and chemical assays.
- Bioassays measure the biological response or activity of the hormone. They can be based on *in vivo*, *in vitro* or synthetic systems.
- Immunoassays (RIA, ELISA) include competitive binding assays and sandwich formats and are based on specific binding of a hormone by a specific antibody.
- Chemical assays include various chromatographic methods (HPLC, GC and electrophoresis).
- Hormone–receptor binding can be measured via the saturation binding assay with Scatchard analysis and the displacement or competition binding assay.
- Specific (low capacity, high affinity) binding of hormone to receptors is separated from non-specific (high capacity, low affinity) background binding.
- Scatchard analysis is used to determine the affinity of hormone binding to its receptor and the effective number of receptors present in a cell.
- Competitive binding experiments measure the ability of a test factor to displace the hormone from its receptor.

The action of hormones is dependent on the concentration of the hormone, whether the hormone is present in its active form (not as a prohormone or bound to a carrier protein), and the presence of sufficient receptors in the target tissue that bind the hormone with high affinity.

Assay of hormones

Assays for hormones are used primarily to measure the levels of hormones in plasma or tissue extracts. They include: bioassays, which measure the activity of the hormone on a target organ (*in vivo*) or target tissue and cells or synthetic expression systems (*in vitro*); immunoassays, which are based on specific binding of a hormone by an antibody preparation; and chemical assays, which are based on functional groups in the chemical structure of the hormone.

All assays must meet several criteria and be validated for the type of sample (species and tissue type) to be of use. The assay should be calibrated with a standard of known activity. The standard must be tested at a number of concentrations to demonstrate that the response in the assay is proportional to the amount of hormone. A plot of the response in the assay versus the concentration of standard is known as a standard curve (Fig. 2.18). The regression equation of the line gives the response factor, which is used to convert the response in the assay that is obtained with a sample to the concentration of hormone in the sample.

The standard and unknown should be measured at the same time and under the same conditions as the samples. There should be near 100% recovery of standard added to extract and this recovery should be consistent between samples. A comparison of standard curves obtained with pure standard in buffer to a sample spiked with standards should show that the curves are parallel. This parallelism of standard curves needs to be validated for each tissue type (for example, plasma will be different from saliva) to demonstrate that the response in the assay is not dependent on the sample matrix (Fig. 2.19). This is especially important in immunoassays using biological samples such as plasma, but should also be accounted for in sample preparation for chemical assays. When the standard curves are not parallel, the samples need to be extracted to remove the interfering material.

Sufficient replicates should be done to demonstrate reproducibility; this is determined by a low coefficient of variation (CV = standard deviation/mean). Quality control (QC) samples representing the low and high ranges of the assay should be included in each batch of samples to assess intra-assay and inter-assay accuracy and precision. The assay must be sensitive, usually in the picogram to nanogram range for plasma hormones. Sensitivity is measured as: the limit of detection (LOD), in which the signal in the assay is three times higher than the background noise (signal-to-noise ratio of 3);

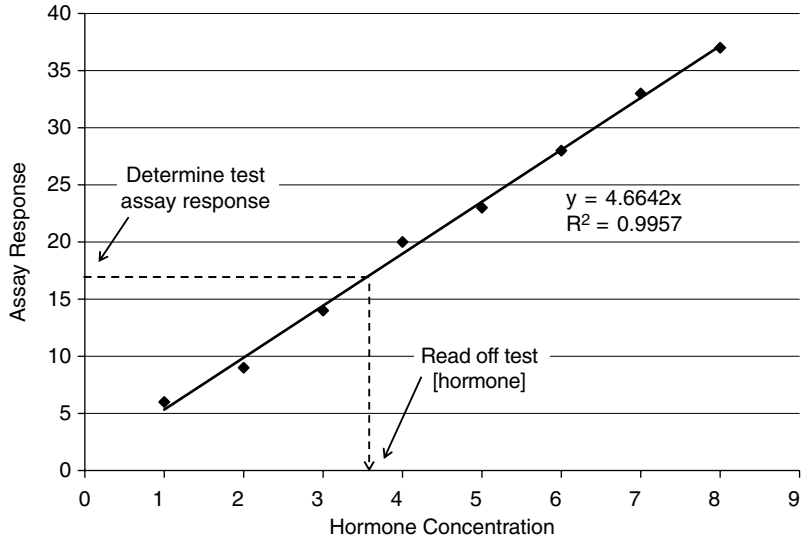


Fig. 2.18. Typical standard curve.

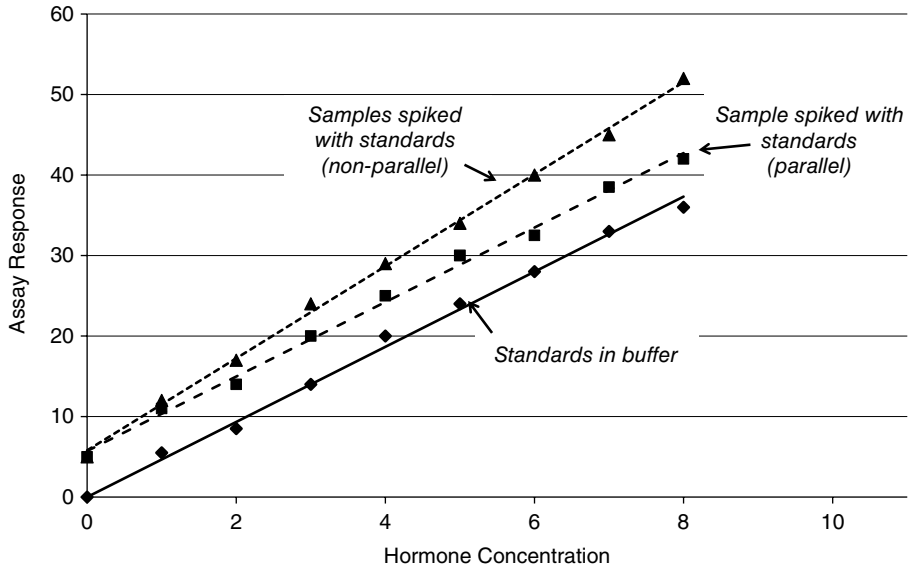


Fig. 2.19. Example of parallelism in standard curves.

and the limit of quantification (LOQ), which is the lowest concentration that can be measured with acceptable accuracy (80–120%) and precision (CV < 20%).

The ideal assay should also be simple to run, low cost and suitable for routine use. The response measured in the assay must also correspond to hormone activity. This is important when the assay is based on structural components of hormones that may not be directly related to activity.

What does all this mean? In order to use an assay to measure a hormone, first determine the response in the assay for different amounts of a pure reference hormone. Construct a standard curve by plotting the response in the assay versus the amount of added hormone and determine the equation of the line. When the response in the assay with an unknown sample is measured, use the equation from the standard curve to determine the amount of hormone in the unknown. Prepare additional standard curves by adding the pure reference hormone to the sample matrix being used, to check for parallelism.

Types of hormone assays

BIOASSAYS The bioassay is the primary method for measuring the amount of active hormone, since it is based on measuring the physiological responses caused by the hormone. Whole animals or tissue preparations utilizing an organ or tissue that is naturally responsive to the hormone can be used. Some examples of bioassays are listed in Table 2.2 (see also Section 6.4 for bioassays for endocrine disruptor chemicals (EDCs)).

The parameter or response measured in the bioassay should be specific for the hormone and quantifiable. This can be a simple positive/negative result, as is the classic pregnancy test, in which urine from the test subject is injected into rats or rabbits and the effect on the ovaries is noted. Human chorionic gonadotrophin (hCG) in the positive test causes ovaries to turn from being normally pale yellow to pink, due to increased blood flow. However, the result should ideally show a dose–response relationship in which the intensity of response is proportional to the amount of hormone. The response should also be directly controlled by the hormone and not dependent on or limited by the presence of other factors for response. Otherwise, these other factors may also contribute to variability in the response parameter. The response should also be easily measured, such as an increase in weight. The parameter can be electrophysiological. For example, in measuring olfaction in fish, the transmembrane potential in neurosecretory neurons in the hypothalamus is recorded with microelectrodes. The magnitude of the electrical

Table 2.2. Some examples of bioassays.

Hormone	Assay system	Responses monitored
In vivo systems		
Insulin	Fasted rodent	↓ Blood glucose
FSH	Immature or hypophysectomized rodent	↑ Weight or follicular size
TSH	Any vertebrate	↑ Thyroid uptake of ^{131}I and release of $[^{131}I]T_4$ and $[^{131}I]T_3$
Thyroxine	Larval amphibian	Metamorphic change
Somatotrophin	Tibia test (rat)	↑ Width of epiphysial (cartilage) plate
Oxytocin	Rat uterus	↑ Contraction
Parathormone	Parathyroidectomized rat	↑ Ca^{2+} in plasma
Oestrogens	Castrated or immature rodent	Vaginal cornification
Androgens	Castrated or immature rodent	↑ Weight of prostate and seminal vesicles
Prolactin	White squab pigeon	↑ Height of crop sac epithelium
Chorionic gonadotrophin	Female rat or rabbit	Increased ovarian blood flow
	Galli–Mainini male frog test	Induced spermiation
	Ascheim–Zondek test, mature rodent or rabbit	Formation of haemorrhagic follicles and corpora lutea
In vitro systems		
Melanotrophins	Frog skin	↑ Darkening of skin, melanosome dispersion
	Melanoma cells	↑ Tyrosinase activity, melanin production
Corticotrophin	Perfused adrenal	↑ Synthesis and release of cortisol

potential is a measure of the response of the fish to various compounds.

The disadvantages of bioassays include the following.

1. A lack of sensitivity, particularly with whole-animal studies, which require a lot of hormone to produce a response.
2. Poor reproducibility, due to the wide variability in response that is obtained with different animals.
3. High cost, difficulty and ethical considerations when animals and animal preparations are used.

In spite of these factors, any type of assay for hormones must be correlated with hormone activity as determined by some sort of bioassay. This makes the bioassay the 'gold standard' against which other assays are compared.

Hormone activity can also be assessed using *in vitro* systems consisting of a cell or tissue preparation that has receptors for the hormone. Binding of the hormone to the receptor and/or activation of an endogenous cellular process such as steroidogenesis, or a gene with 'reporter' activity that is easily measured is assessed (see Sections 1.3 and 6.4). Examples of this approach are the oestrogen receptor coupled to luciferase, which is an enzyme from the firefly (Lampyridae) that produces a fluorescent product. This system has been used to measure binding of oestrogenic 'endocrine disruptor' compounds in the environment to the oestrogen receptor to assess their potential biological activity (see Section 6.4). Similarly, the growth hormone secretagogue receptor (GHS-R) (see Section 3.4) coupled to a fluorescent reporter gene, aequorin, has been used to screen for compounds that bind to the GHS-R. This approach is particularly useful in measuring binding between two different receptors for a particular hormone. For example, corticotrophin-releasing hormone (CRH) acts via two classes of receptors, CRHR1 and CRHR2 (see Section 6.3). These receptors have been cloned and expressed in cells in culture to be used as a bioassay system to identify compounds that bind specifically to one form of the receptor. These types of *in vitro* assays are used in drug discovery work to measure biological activity in a library of compounds that share a core 'privileged structure' that is essential for this activity (see Section 2.3).

Bioassays are also useful for detecting novel biologically active compounds in natural products or complex mixtures. These compounds cannot be measured by highly sensitive chemical assay methods such as gas chromatography or high-performance liquid chromatography–mass spectrometry (see below), because their chemical structure is not known. However, bioassays can detect these active compounds,

since they are not reliant on knowing the chemical structure but instead are based on biological activity. Bioassays are also useful in the purification of novel active compounds from complex mixtures by assessing the effectiveness of different steps in the purification process.

IMMUNOASSAYS Immunoassays are based on specific binding of a hormone by an antibody. They include competitive binding assays, the non-competitive sandwich assay and a number of rapid assay formats.

In the competitive binding assay, a fixed amount of labelled hormone (H*) and a fixed amount of antibody (Ab) are used, along with a variable amount of unlabelled hormone (H). An equilibrium is established between the amount of unlabelled hormone bound to the antibody (H–Ab) and the amount of labelled hormone that is bound to the antibody (H*–Ab) (Fig. 2.20).

The equilibrium position depends on the amount of H present. The amount of H*–Ab decreases as the amount of H in the assay increases, because there is a fixed amount of H* and a fixed amount of Ab in the assay. Since we can only measure the amount of labelled H*–Ab, the amount of H is deduced from measuring the amount of H*–Ab. The response in the assay standard curves from H*–Ab is thus higher when the amounts of H are low, so there is a negative relationship between the amount of hormone in the sample and the response in the assay (Fig. 2.21).

The procedure involves first preparing standard curves to determine the useful concentrations of antibody. A series of different standard curves are produced using a fixed amount of antibody for each curve, with the same amount of labelled hormone throughout, and adding various amounts of unlabelled hormone at concentrations that are relevant for the samples to be assayed. The percentage of bound labelled hormone is determined for each amount of unlabelled hormone in the assay by separating the bound hormone from the free hormone (see below). The percentage of bound labelled hormone is then plotted against the concentration of unlabelled hormone. The amount of antibody that gives a moderate slope from 10% to 90% saturation is used for subsequent assays (Fig. 2.21).

A standard curve is then generated using the appropriate concentration of antibody, with the % bound labelled hormone on the *y*-axis plotted against the concentration of unlabelled hormone on the *x*-axis (log scale). The hormone concentration in a test sample is determined by adding the sample to the antibody and labelled hormone mixture. The amount of bound labelled hormone is measured and the hormone concentration can be determined from the regression equation of the standard curve (Fig. 2.21).

Competitive binding assays require specific, high-affinity binding of hormone and the absence of non-specific interfering substances in the assay. Sometimes there are substances present in physiological fluids that affect binding of hormone to the antibody, and it may be necessary to partially purify the hormone and remove the interfering substances before assay. This can be determined by checking for parallelism of standard curves, as described above (see Fig. 2.19).

Several types of binding proteins aside from antibodies can potentially be used in competitive binding assays. Hormones bind to receptors with high affinity and specificity and it is possible to use target tissue plasma membrane or intact cells as a source of binding protein. The hormone binds to its own receptor and the specificity of binding can be checked by competition with other hormones to replace labelled hormone. It is also possible to determine the number of receptor binding sites if a known concentration of hormone is used. This is known as Scatchard analysis (see below). Proteins present in blood serum, such as specific hormone-binding globulins, can also potentially be used in competitive binding assays, but the binding affinity and specificity are usually not adequate.

Another alternative to using antibodies is aptamers, which are short sequences of single-stranded

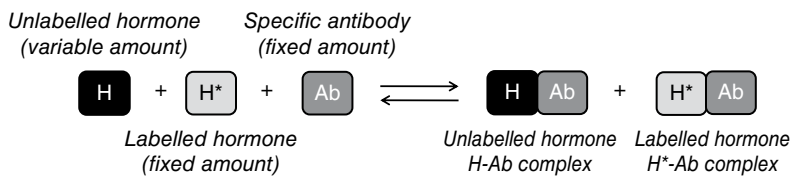


Fig. 2.20. Equilibrium in competitive binding assays.

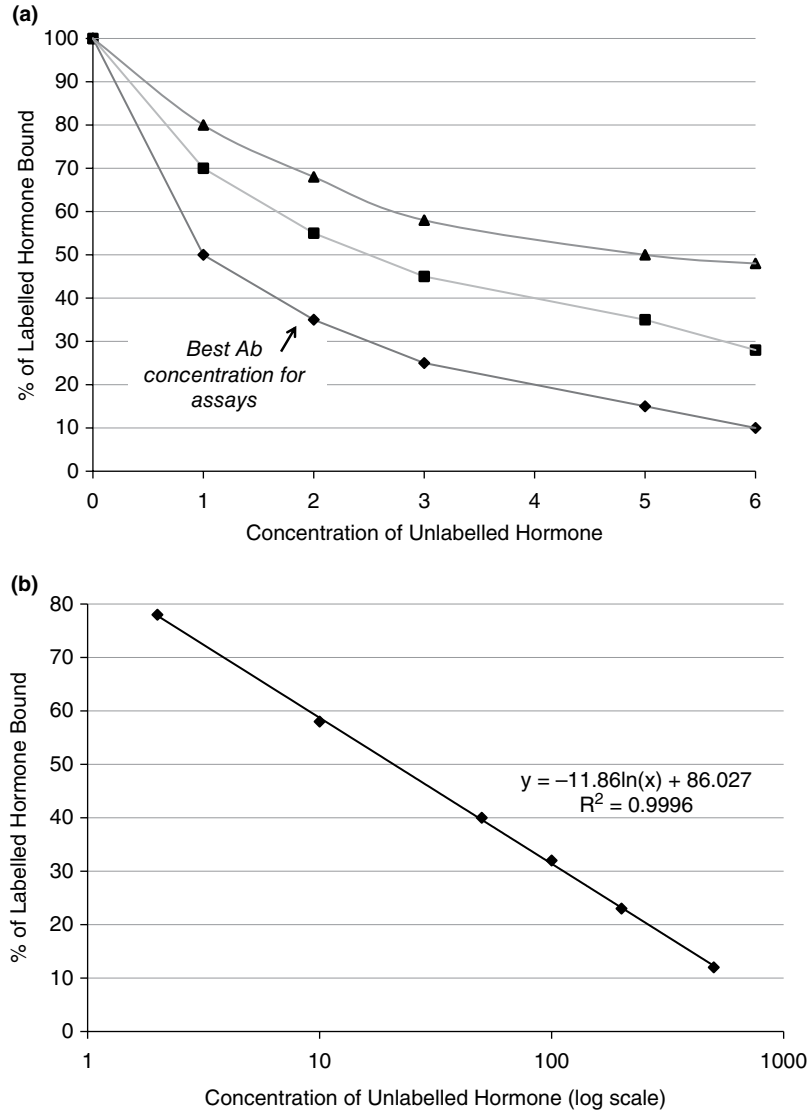


Fig. 2.21. Standard curves for competitive binding assays. **(a)** Binding curves using different amounts of binding proteins. **(b)** Typical standard curve for assay.

DNA or RNA molecules that bind target molecules with high affinity (see Fig. 2.17 in Section 2.1). They are more stable than antibodies, can be labelled with various dyes and can be synthesized easily and at low cost.

Most often, specific antibodies generated by immunizing an animal with hormone are used as binding proteins, as in immunoassays. Antibodies to steroids are generated using the steroids conjugated to an antigenic protein at a site on the steroid that leaves the unique functional groups on the steroid available as epitopes for antibody production. Most commercially available immunoassay kits use monoclonal antibodies (see Section 2.1) since this system can produce large amounts of highly specific antibody of consistent quality.

Competitive binding assays require efficient methods to separate bound hormone from free hormone. These methods must not change the binding equilibrium and should be simple and efficient so

that a number of samples can be processed at the same time. There are several methods available.

1. A second antibody can be used to precipitate the primary antibody–hormone complex (Fig. 2.22). The antibody–hormone complex is collected by centrifugation, leaving unbound hormone in solution.
2. The free hormone can be adsorbed on a solid-phase material such as charcoal with a coating of albumin or dextran. The charcoal is removed by centrifugation and the hormone bound to antibody remains in solution.
3. Bound and free hormone can also be separated using column chromatography with gel filtration media such as Sephadex; convenient spin columns are available for separating different-sized molecules.
4. A most convenient way of separating bound from free hormone is to link the antibody to a solid support, such as glass beads, magnetic beads, filter paper or the assay tube. This latter format is referred to as a solid phase assay (Fig. 2.22). The labelled hormone that is not bound to the antibody is removed by simply decanting the tube or collecting the beads with the bound antibody.

Labels used for immunoassays can be radioactive, fluorescent or an enzyme that produces a colour change. A major assumption is that the labelled hormone has the same binding characteristics to the antibody as the unlabelled hormone. A radioimmunoassay (RIA) can use steroids labelled with ^{14}C or ^{3}H; I^{125} can be used to label steroids or tyrosine residues of protein by mild oxidation. The amount of bound labelled hormone is determined by counting the radioactivity. A fluorescence immunoassay (FIA) uses a fluorescent label such as fluorescein isothiocyanate, which has an excitation maximum at 485 nm and an emission maximum at 525 nm. In the enzyme-linked immunosorbent assay (ELISA or EIA), an enzyme such as alkaline phosphatase or horseradish peroxidase is coupled to the hormone (Fig. 2.23). The enzyme catalyses the hydrolysis of substrates that produce a coloured product, such as BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium) for alkaline phosphatase and TMB (3,3′,5,5′-tetramethylbenzidine) for peroxidase. The extent of colour change is used to quantitate the amount of labelled antibody–hormone complex that is present. The signal amplification from these chromogenic substrates can increase the sensitivity of ELISA.

As an alternative to the competitive immunoassays, an antibody sandwich ELISA can be used to measure hormone concentrations (Fig. 2.24). This

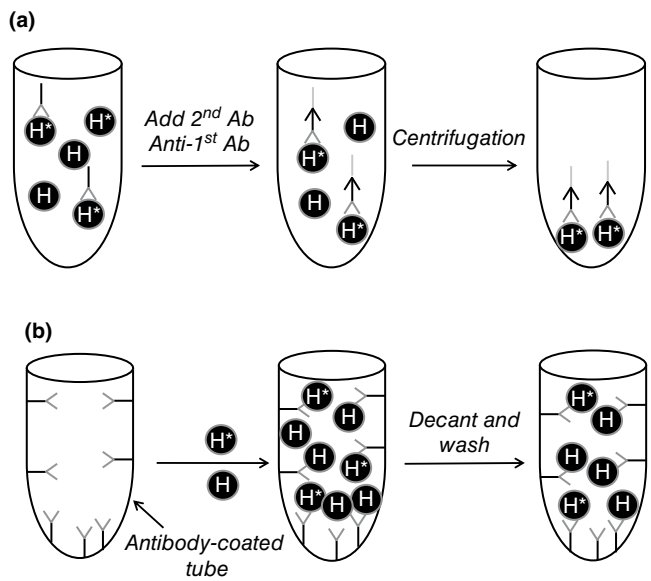


Fig. 2.22. Methods for separating bound hormone from free hormone. **(a)** Precipitation with 2nd Ab against 1st Ab, followed by centrifugation. **(b)** Antibody fixed to solid support.

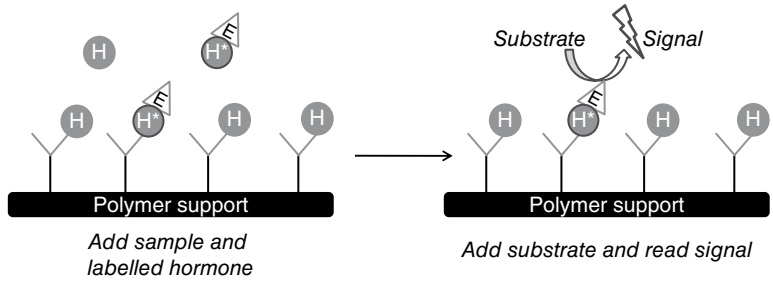


Fig. 2.23. Format for competitive ELISA.

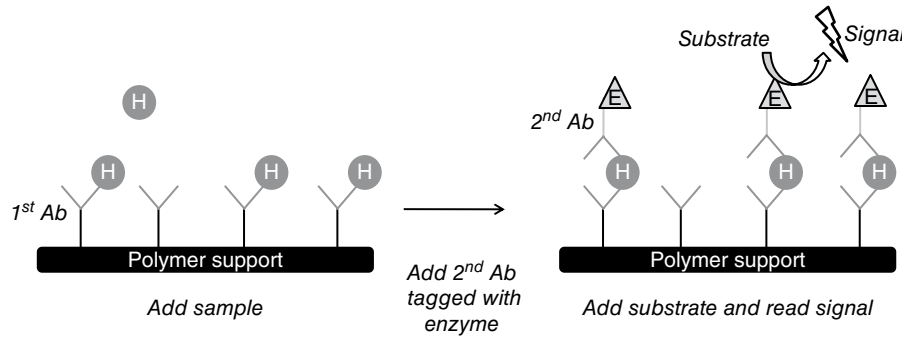


Fig. 2.24. Format for sandwich ELISA.

assay uses two antibodies specific to different sites on the hormone. In this format, a primary capture antibody is bound to a solid support (the tube or microtitre plate) and incubated with unlabelled hormone (standard or in a sample). This is then followed by incubation with a second indicator antibody against the hormone, which is conjugated to an enzyme. The activity of the enzyme is then measured using substrates that produce a coloured product, as for the competitive ELISA. The amount of hormone bound to the capture antibody is estimated from the enzyme activity on the bound indicator antibody. The sandwich ELISA format does not have a competition for binding to the antibody by labelled hormone, and the signal in the assay increases with the amount of hormone in the assay. Thus, there is a positive relationship between the signal in the assay and the amount of hormone in the sample. (Recall that there is a negative relationship between the amount of hormone in the assay and the signal in the assay in a competitive binding immunoassay.) The lack of competition in the sandwich ELISA also makes this format useful for detecting low levels of hormone or when many contaminants are present in the sample that can affect competitive binding.

There are a number of rapid ELISA formats available for high-throughput analysis of various compounds and these can be divided into lab-based instrumental methods and non-instrument methods for on-site testing outside the laboratory.

Instrumental methods for use in the laboratory to obtain quantitative results include microtitre plate ELISA, different kinds of immunosensors, fluorescence polarization immunoassay and capillary electrophoretic immunoassay. The typical microtitre plate format of 96 wells allows for the analysis of multiple samples simultaneously or the analysis of multiple compounds by using antibodies for different compounds in separate rows on the plate. Immunoassays are commercially available for many hormones. However, most of these kits are designed for use in humans or rodents, so they must be validated before being used for analysis of hormones in livestock species. Immunosensors are devices that change physiochemical characteristics when biological molecules interact, as when an antigen binds to an antibody, and this generates an

electrical signal. There are three main groups of sensors: colorimetric/luminescent sensors, electrochemical sensors and surface plasmon resonance sensors. Colorimetric and luminescent sensors measure visible or ultraviolet light produced by the antigen–antibody complexes, while electrochemical sensors measure the production of an electrochemically active product. Surface plasmon resonance is based on changes in oscillations of free electrons (plasmons) on the surface of thin metal films after binding of compounds. This is detected by changes in the resonance angle at which a light beam is absorbed at the metal surface. This approach does not require special labels on the antigens or antibodies for detection. The fluorescence polarization immunoassay is based on the concept that unbound fluorescently labelled hormone has a higher rate of rotation in solution and consequently a lower fluorescence polarization value than labelled hormone bound to an antibody. Therefore, the fluorescence polarization value is inversely proportional to the amount of unbound labelled hormone and there is no need to separate free and bound hormone. In the capillary electrophoretic immunoassay, the free and bound hormone are separated by electrophoresis of a few nanolitres of sample on capillary tubes. As the amount of hormone in the sample increases, the signal from the free labelled hormone increases and the signal from the bound labelled hormone decreases.

Non-instrument methods for use outside the lab for on-site testing give qualitative visual results as positive or negative, based on a set cut-off level. These tests can be convenient and simple but generally are not as sensitive as the microtitre plate ELISA format. They include lateral flow test or immunochromatographic strip (ICS), dipstick and flow-through tests. The lateral flow strip consists of three parts: a conjugate pad which contains labelled antibody; a porous membrane containing the test line with secondary reagents and a positive control line; and an absorbent pad (Fig. 2.25). Sample is applied on the conjugate pad and the antibody and antigen–antibody complex move along the membrane by capillary action towards the absorbent pad. If the complex is present, there is a signal at the test line, while the control line detects the presence of the antibody to confirm that the test was completed. The sensitivity (cut-off level) is set by adjusting the concentration of the reagents used. Lateral flow test strips have been developed for the detection of clenbuterol, a β-adrenergic agonist (see Section 3.7) and for human chorionic gonadotrophin (hCG) used in pregnancy tests. For more information on lateral flow assays, see the review by Koczula and Gallotta (2016).

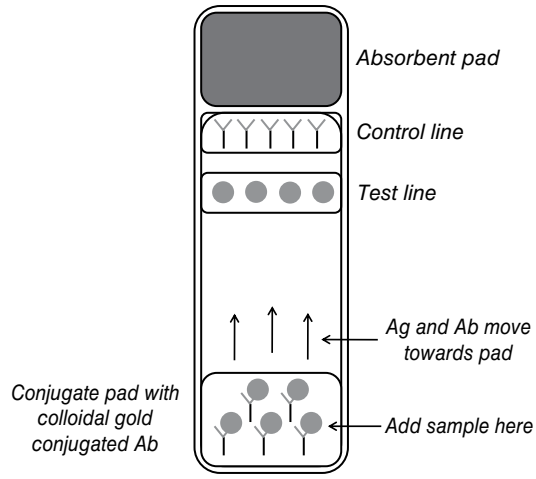


Fig. 2.25. Format for lateral flow rapid ELISA.

The dipstick format uses a strip containing a specific antibody that is placed in different solutions containing the sample, the labelled hormone and the substrate to produce a coloured product. Flow-through or immunofiltration tests also use a membrane with specific antibodies attached, but the membrane is placed on an absorbent pad to prevent back-flow of fluids. The various components are added to the membrane in turn to finally develop a coloured product.

CHEMICAL ASSAYS Chemical assays for hormones are based on some aspect of the structure of the particular hormone and are especially useful when there is no immunoassay or bioassay available. Sometimes it is possible to take advantage of unique aspects of the hormone structure to design a simple and specific measure. For example, one assay method for thyroxine is to measure total protein-bound iodine. Uptake of labelled iodine can also be used to measure biosynthesis of thyroxine in the thyroid gland. However, chemical assays that test for functional groups on the hormone molecule that may not be involved with activity can give false results compared with biological activity.

The solubility and stability of the hormone are related to its chemical nature. If the hormone is soluble in organic solvents, it is probably a steroid,

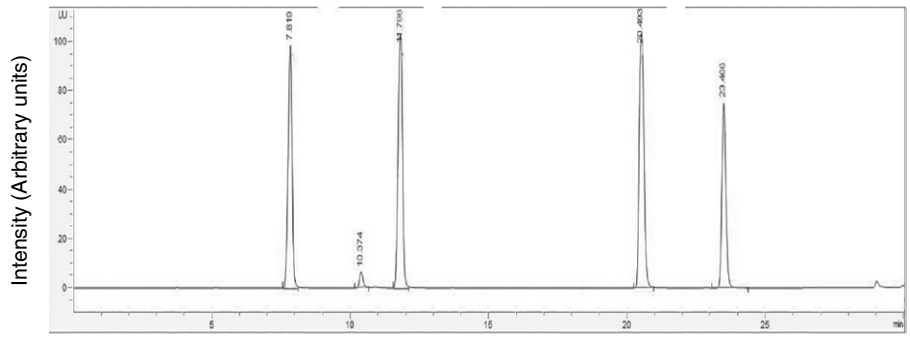
lipid derivative, or other hydrophobic molecule, while if it is soluble in water, it is more polar and probably a protein or amino acid derivative. Protein hormones can also be inactivated by heat, changes in pH or digestion with proteases, while steroid hormones are stable to these treatments. These solubility and stability tests can be used in determining the chemical nature of an unknown hormone activity.

One form of chemical assay involves purifying the hormones by chromatography and then measuring the amount of hormone. Separation of compounds by chromatography depends on the differential partitioning of the compounds between the stationary phase (the material in the column) and the mobile phase that is flowing through the column. It is important to prepare and extract the sample before chromatography to remove interfering compounds that can affect the chromatographic separation or block the column.

LIQUID CHROMATOGRAPHY Purification by chromatography can be done using open-column chromatography, but high-performance liquid chromatography (HPLC) is more commonly used as an analytical technique. HPLC uses high-resolution columns containing very uniform particles. These columns can separate very similar molecules with high efficiency and normally operate under high pressure. The outflow from the column is connected to a detector, which measures some chemical characteristic of the column effluent, such as the absorbance of ultraviolet or visible light, fluorescence, refractive index, conductivity, radioactivity or even molecular mass (using a mass spectrometer). A typical chromatogram is shown in Fig. 2.26. Note that a number of different compounds can be separated and quantified in one chromatographic run. Each compound is identified by its retention time, which is the time after sample injection that the compound elutes from the column. The amount of each compound is determined by the area under the peak obtained for that compound. The system is calibrated for the retention time and relative peak area using known standards for each compound. The typical arrangement of a chromatography system is shown in Fig. 2.27.

The types of chromatography columns include the following.

1. Ion exchange (either anion or cation), which separates molecules based on charge.
2. Adsorption (normal phase) chromatography, which is similar to thin-layer chromatography in that it works by surface adsorption of molecules to silica and is used to purify small organic molecules.
3. Hydrophobic interaction (reverse phase), which separates by relative polarity or hydrophobicity



Interpretation of Chromatography Results

Peak #	AREA%	RT	AREA
1	23.74	7.82	1002
2	1.36	10.37	57.5
3	27.51	11.80	1161
4	29.31	20.49	37
5	18.08	23.47	763.3

Fig. 2.26. A typical chromatogram from a chromatography system. RT, retention time.

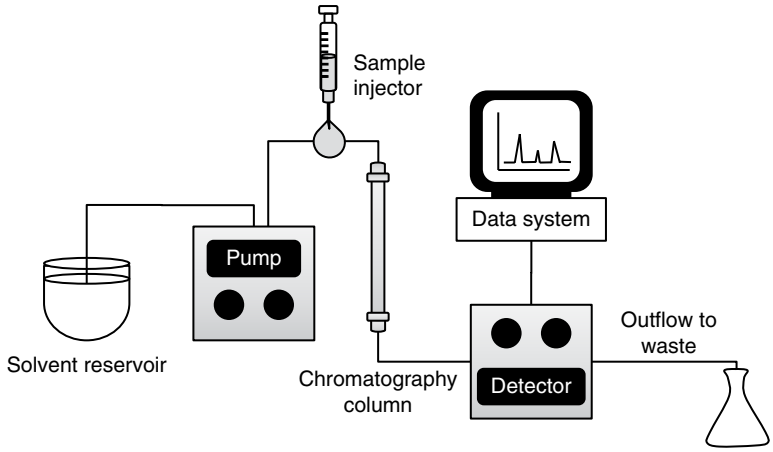


Fig. 2.27. Typical chromatography system.

with the polar compounds eluting first from the column. Common reverse-phase columns are C18 and C4, which are used for separating small hydrophobic molecules such as steroids, drugs and amino acids, and phenyl, which is used for proteins.

4. Size exclusion or gel filtration, which separates molecules based on their size and shape.
5. Affinity chromatography, in which the compound of interest binds to a specific ligand attached to the column matrix, giving a one-step purification.

In ion-exchange, normal-phase and reverse-phase chromatography, separation of the various compounds is achieved by changing the composition of the mobile phase over time. The compounds that are tightly bound to the column are removed by changing the pH, ionic strength or percentage of organic solvent in the mobile phase, usually as a gradient. A group of compounds with a similar chemistry can also be separated using an isocratic system with no changes in the mobile phase during the chromatographic run. In size-exclusion chromatography, the small molecules enter the pores in the gel matrix and are retained on the column, while the larger molecules are excluded from the gel and elute first in the initial void volume. Gel filtration is usually not high enough resolution for analytical work but is useful in preparative work for large molecules such as proteins. An affinity column can be made by attaching an antibody or binding protein to the column matrix and then applying a crude hormone preparation to the column. The hormone binds to the protein on the column and the other compounds pass through the column. The pure hormone is then eluted with a high salt or pH gradient. Affinity chromatography can also be used to purify antibodies (see Section 2.1.). The types of liquid chromatography systems are summarized in Table 2.3.

GAS CHROMATOGRAPHY Gas chromatography (GC) separates compounds by adsorption or partitioning between the mobile gas phase and the liquid or solid stationary phase. The partitioning is dependent on temperature, so the temperature is increased during the run to elute the low-boiling compounds first and the high-boiling compounds last from the stationary phase. Compounds must be volatile to be separated by GC, so charged groups are normally derivatized to make them less polar. Common detectors for GC are thermal conductivity (TCD) and flame ionization detectors (FIDs). The FID is sensitive over a large range of concentrations but destroys the sample. With the FID, the effluent from the column passes through a hydrogen/air flame that produces ions from organic molecules present in the effluent. The ions are collected on an electrode to produce an electrical signal. With the TCD, organic molecules in the column effluent change the thermal conductivity of the carrier gas, which changes the resistance of an electrically heated wire or thermistor. This change in resistance is converted to an electrical signal. The TCD is not as sensitive as an FID but does not destroy the sample.

Mass spectrometry can be used to determine the molecular weight, structure and position of functional

Table 2.3. Liquid chromatography systems.

Type	Separation principle	Usage
Ion exchange	Net charge	Proteins, charged molecules
Reverse phase	Relative hydrophobicity	C18 – small hydrophobic molecules Phenyl – proteins
Normal phase	Surface adsorption	Small organics
Size exclusion	Molecular size and shape	Large molecules (proteins, etc.)
Affinity	Specific binding affinity	Unlimited

groups of small molecules. The mass spectrometer produces charged ions from the sample, consisting of the parent ion and fragments of the original molecule, and then sorts these ions by mass/charge ratio in a magnetic field. The relative numbers and the mass/charge ratio of each ion are characteristic of a particular compound and can be compared with a library of mass spectra to identify the structure of the compound. A mass spectrometer can be linked to a GC (GC–MS) or HPLC (HPLC–MS) to identify the compounds that are separated by the chromatography.

ELECTROPHORESIS Proteins can be separated by electrophoresis, usually on polyacrylamide gels. Native or non-reducing gels separate intact protein molecules based on charge and size, while gels containing sodium dodecyl sulfate (SDS) separate molecules based on their molecular weight. SDS coats proteins and gives them an overall negative charge, so the migration of the protein in the gel is affected by the molecular weight and not the charge of the protein. After electrophoresis, the gels are stained to visualize all the proteins. The proteins can also be transferred or blotted to a solid membrane support and stained with a specific antibody that recognizes the protein of interest. This is known as Western blotting. A protein band can also be cut from a gel, the protein eluted from the gel fragment and a partial amino acid sequence determined. This information can be used to identify the protein and to produce DNA oligonucleotide probes as a first step in cloning the protein (see Section 2.3). Capillary electrophoresis is a high-resolution analytical method useful for separating a variety of compounds. It uses just a few nanolitres of sample for capillary tubes with internal diameters of 20–100 μm.

Measurements of hormone–receptor binding

Hormones bind to receptors with high affinity (dissociation constant of 10^{-9} M) and receptors are normally highly specific for binding a particular hormone. There are two main types of assays used to study hormone–receptor binding: the saturation binding assay with Scatchard analysis and the displacement or competition binding assay. The requirements for receptor binding assays include a hormone standard that is able to bind the receptor, receptors that are present in their native (active) form and a method to detect and measure bound hormone, which is generally achieved using hormone with a radioactive or fluorescent label. For cell surface membrane receptors, either whole cells or a subcellular membrane fraction can be used, while for intracellular receptors, the nuclear fraction can be used.

Scatchard analysis is used to determine the affinity of hormone binding to its receptor and the effective number of receptors present in a cell. Measuring the affinity of hormone binding to its receptor is complicated by the binding of hormone to nonspecific sites that occurs at the same time. Therefore, it is necessary to separate specific (low capacity, high affinity) binding of hormone to receptors from non-specific (high capacity, low affinity) background binding of hormone to other components of the system, such as other sites on the cell or the assay vessel (Fig. 2.28). Non-specific (NS) binding is high capacity since there are many sites where NS binding can occur, so saturation is unlikely; the more ligand added, the more NS binding is detected. Specific binding is low capacity, due to the limited number of receptors present, and reaches a saturation point; this is the maximum binding (B_{max}), which is a plateau that is reached when all receptors are occupied (see Fig. 2.29).

In the saturation assay, non-labelled (H) and labelled (H*) hormones compete for binding to the receptor (R). The affinity of binding should be identical for (H) and (H*). After equilibrium is reached, free labelled hormone is removed before counting the amount of bound hormone.

A saturation binding curve is first generated by incubating increasing amounts of labelled hormone (H*) with a receptor (R) preparation, with no

unlabelled hormone (H) present (Fig. 2.29). The bound hormone is separated from the free hormone and the amount of hormone binding is measured. This corresponds to total binding, which includes specific binding to the receptors (H*–R) and non-specific (NS) binding of hormone (H*–NS).

The next step is to determine the H*–NS binding. This is done by running a second series of assays that includes a large excess of unlabelled H (100–1000 times excess) in the assay to saturate the limited number of specific receptor sites. When labelled H* is added in the presence of this excess of H, there are no specific receptors available for binding H*, so it can now only bind to the large number of non-specific sites available and this corresponds to non-specific binding.

The specific binding is then calculated as the difference between the total binding and the non-specific binding (specific binding = total binding – NS binding) at each amount of labelled H* in the assay (Fig. 2.29). When all the receptors are occupied, a plateau or saturation point is reached and this represents the B_{max}, which is a measure of the number of receptors.

The binding of hormone (H) to its receptor (R) to form a hormone–receptor complex can be described by the equation:

$$R + H \leftrightarrow RH$$

The equilibrium association constant K_a and the dissociation constant K_d are defined as:

$$K_a = [RH]/[R][H] = 1/K_d$$

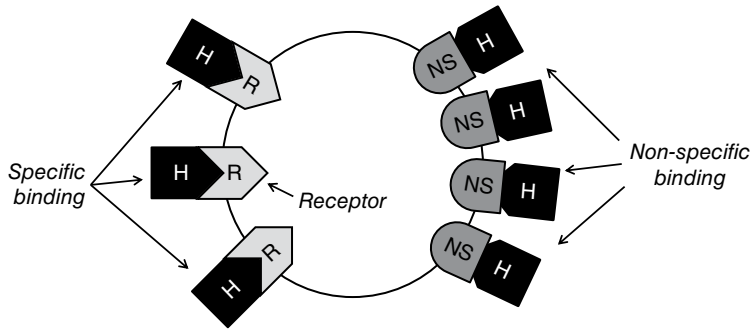


Fig. 2.28. Specific binding and non-specific binding.

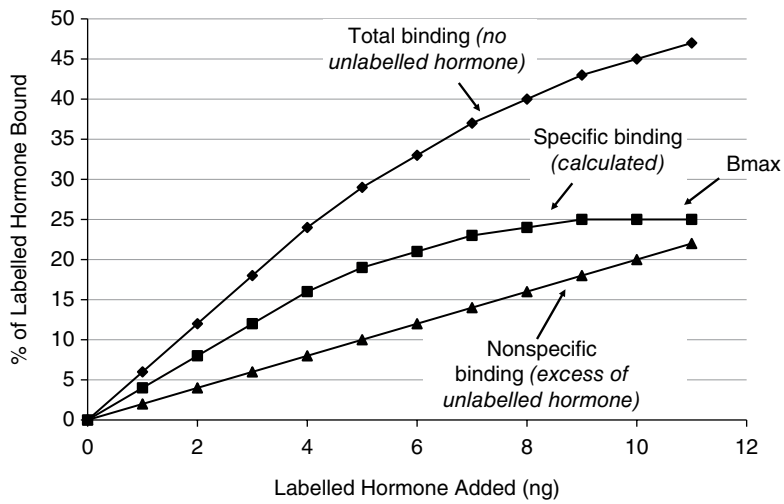


Fig. 2.29. Saturation binding curves for hormone–receptor binding.

The ratio of bound (RH) to free (H) hormone (B/F) is given by:

$$B/F = [RH]/[H] = K_a[R]$$

The total number of receptors (Rt) is given by:

$$[Rt] = [R] + [RH]$$

Combining these equations:

$$B/F = K_a([Rt] - [RH]) = -K_a[RH] + K_a[Rt]$$

The Scatchard plot equation is:

$$B/F = K_a[Rt] - K_a[B]$$

The specific binding data from the saturation binding curve are plotted (Fig. 2.30), comparing the ratio of specifically bound to free hormone (*y* variable) versus hormone specifically bound to the receptor preparation (RH or B) (*x* variable). The slope of the line is $-K_a$, the *x* intercept is Rt (the total number of receptors, determined when all the receptors are occupied) and the *y* intercept is K_a [Rt]. This is the Scatchard plot, which corresponds to a linear representation of the saturation curve.

The dissociation constant for the RH complex (K_d) is a measure of the binding affinity of the hormone for the receptor. When the concentration of hormone equals the K_d, one-half of the receptors are occupied. A small K_d indicates a high affinity of the receptor for the hormone, since a low amount of hormone is needed to get a response. The K_d for steroid receptors is in the range of 10^{-8}–10^{-10}. As an approximation, 20 times the K_d is enough to saturate the receptor. At the *x* intercept, all of the hormone is in the bound form since the bound/free ratio = 0, and this indicates the number of receptors present (Rt).

A variation in binding affinity or receptor cooperativity is seen when binding of hormone to one receptor affects binding of subsequent hormone molecules. This is seen as a curve in the Scatchard plot, since a change in affinity would result in a change in slope of the plot. Positive cooperativity indicates that the initial binding of hormone enhances further binding and is seen as an upward

For interest

Example of Scatchard analysis (Fig. 2.30)
In general, the amount of specific binding (H*–R) is much smaller than the amount of free hormone (H*). For analysis, it is assumed that the free H* at equilibrium is equivalent to the initial amount of H* added, which requires total binding to be always less than 10% of the initial radioactivity.

Bound	Free	Bound/Free
4	2000	0.003
10	5000	0.002
15	10000	0.0015
20	15000	0.0012
22	20000	0.001

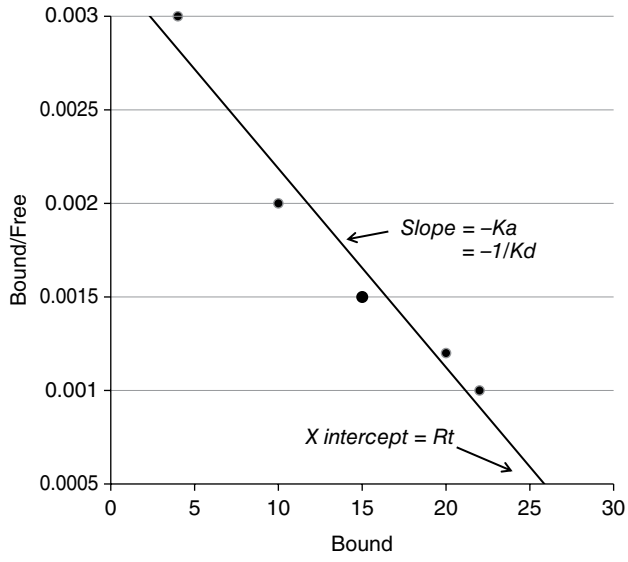


Fig. 2.30. Example of Scatchard analysis of hormone–receptor binding.

curve in the Scatchard plot. This may be important in sensitizing a system for a hormone response. Negative cooperativity is seen when the initial binding reduces further binding and produces a downward curve in the Scatchard plot. It may provide a mechanism for desensitizing a tissue to abnormal hormone levels. This is seen with receptors for insulin, TSH and nerve growth factor. In cases where two types of receptors bind the same hormone with different affinity, this results in a non-linear Scatchard plot.

The regulation of receptor numbers represents another endocrine control mechanism (see Table 1.6). A number of factors affect the receptor number, including cellular development, differentiation and age. There can be either up-regulation or down-regulation of receptor numbers. Hormones can induce synthesis (up-regulation) of their own receptors; for example, prolactin induces liver receptors. Insulin down-regulates its receptors in lymphocytes. Binding of hormone can stimulate the internalization of the hormone–receptor complex, thus reducing the available binding sites and desensitizing the cell. Changes in receptor number and affinity can be determined by Scatchard analysis. When one hormone regulates the receptor number of another hormone, this is heterospecific regulation. This is a mechanism for hormones to act in sequence to amplify or diminish response to other hormones. Homospecific regulation occurs when a hormone regulates levels of its own receptor; this prevents hyperstimulation of cells in pathological states.

The displacement or competitive binding assay is based on the binding competition between labelled hormone and a different but related unlabelled test factor; it measures the ability of the test factor to displace the hormone from its receptor. A single concentration of labelled hormone is incubated with receptor along with increasing concentrations of the test factor F, resulting in displacement of the equilibrium and reduced binding of labelled hormone to its receptor. The ability of factor F to compete for binding with the labelled hormone (H*) is measured by separating the free labelled hormone from the bound labelled hormone and counting the bound hormone. The K_d for factor F is calculated by:

$$K_{d(F)} = IC_{50(F)} / (1 + [H^*] / K_{d(H)}$$

where $IC_{50(F)}$ is the concentration of F that displaces 50% of the bound H*. Most factors will not compete with H* for binding, as the binding of hormone to receptor is very specific and high affinity. This assay is useful for comparing binding of hormone analogues to a receptor, since useful hormone analogues will also bind the receptor with high affinity. A competitive binding experiment of potential endocrine disruptor chemicals (EDCs) (see Section 6.4) measuring the displacement of testosterone (T) from the AR and oestradiol (E_2) from the ER is shown in Fig. 2.31.

For interest

Compare and contrast how labelled and unlabelled hormone are used in immunoassays to assay for hormones and in receptor binding assays for Scatchard analysis.

2.3 Methods for the Production of Hormones

Key concepts

- The structure of steroid hormones is the same in all species. More potent agonists or antagonists of steroids have been chemically synthesized.
- The structure of a particular peptide hormone can vary among different species, with some homology between close relatives.
- Peptides can be sequenced by Edman degradation and MALDI–TOF mass spectrometry or the protein sequence can be deduced from the cDNA sequence.
- Peptides can be made by Merrifield synthesis using a solid-phase support or by expressing longer peptides in bacteria, eukaryotic cells or transgenic animals.
- Peptidomimetics that mimic the structure of peptide hormones have been developed as orally active agonists or antagonists.
- Proteins can be made using recombinant DNA techniques *in vitro,* in bacteria or eukaryotic cells, or *in vivo* using transgenic animals.

One of the common methods for manipulating endocrine function is to treat animals with a source of exogenous hormones. Reliable and inexpensive sources of hormones are needed to

provide the hormones that can be administered to animals. Very early work used tissue extracts, which were very crude and contained a mixture of hormones, degradation products and other contaminants. In later work, the hormones were isolated and purified and their structure was determined, so that usable amounts of the hormone could be synthesized. For some hormones, alternative sources were found, such as plant hormone agonists, or the hormone could be synthesized from natural-source precursors either chemically or using microbial bioreactors. More potent analogues of steroid and peptide hormones have been developed.

Steroids and non-protein hormones

For interest

Historical aspects of hormone purification and identification

Epinephrine was isolated by Oliver and Shafer from adrenal glands and then synthesized in 1895. In 1919, thyroxine was purified by Kendal from thyroid glands of swine; in 1926, Harrington showed it to be a derivative of tyrosine. Oestrogen was the first steroid isolated; Doisy and Butenandt did this from human pregnancy urine. Later, Doisy, in 1935, and MacCorquodale, in 1936, isolated 12 mg of oestradiol-17β from 4 t of sows' ovaries. In 1931, Butenandt isolated androsterone from male urine and, in 1934, progesterone was isolated from sow ovaries by four different groups. In 1935, testosterone was isolated from testes by David and co-workers, who demonstrated that it could be synthesized from cholesterol. In the period 1936–1942, adrenal hormones were isolated by many workers in the USA and Europe. From 20,000 slaughter-house cattle, 100 kg of adrenal glands were obtained and used to isolate 300 mg each of 29 steroids. Hench and co-workers demonstrated that cortisone could be partially synthesized and used it to clinically improve rheumatoid arthritis. See Hadley and Levine (2006).

Microorganisms such as *Rhizopus* and *Aspergillus* can metabolize steroids to useful compounds, and these can be used as bioreactors for the synthesis of steroids. Natural sources of steroids include plant steroids, which are usually conjugated to sugar residues, and bile salts. Hyodeoxycholic acid from bile salts of pigs has been used to synthesize progesterone and testosterone.

Steroids are all based on the same cyclopentanoperhydrophenanthrene nucleus consisting of four fused rings, labelled A, B, C and D, and can be members of either the pregnane, androstane or oestrane families (Fig. 2.32). Different steroids in these families have different functional groups or double bonds on these four rings. Unlike protein and peptide hormones, the structures of steroids are identical across species. The trivial names and systematic names of common steroids are given in Table 2.4.

Synthetic steroid hormone agonists and antagonists have been chemically synthesized (Fig. 2.33). Hormone antagonists bind to receptors but do not cause the normal endocrine response. Hormone agonists have two important properties that increase their potency:

1. They can bind to receptors with high affinity to initiate the endocrine response; this can be measured with receptor binding assays.
2. Agonists can produce a longer-lasting endocrine response since they are degraded more slowly; for example, diethylstilboestrol (DES) is a long-lasting oestrogenic compound.

Ethinyl oestradiol and norethindrone are used as contraceptive steroids, dexamethasone is a synthetic glucocorticoid, and promegesterone (R5020) is a synthetic progestogen. Other compounds are receptor antagonists. Clomiphene and tamoxifen bind to oestrogen receptors to block them. Tamoxifen has also been shown to have oestrogenic effects. Mifepristone (RU-486) is a progesterone antagonist that is used clinically to induce abortions.

Protein and peptide hormones

Protein and peptide hormones are named based on their endocrine activity rather than their chemical structure; for example, growth hormone is thus named because one of its effects is to stimulate growth. The structure of a particular peptide hormone can vary among different species, with some homology between close relatives. Some small peptides are identical; for example, GnRH is identical among mammals but different in chickens and fish. On the other hand, a particular steroid hormone has the same structure regardless of the species in which it is found. For example, growth hormone from pigs and cattle are different, since they have different amino acid sequences. However, progesterone isolated from pigs or cattle is the same molecule.

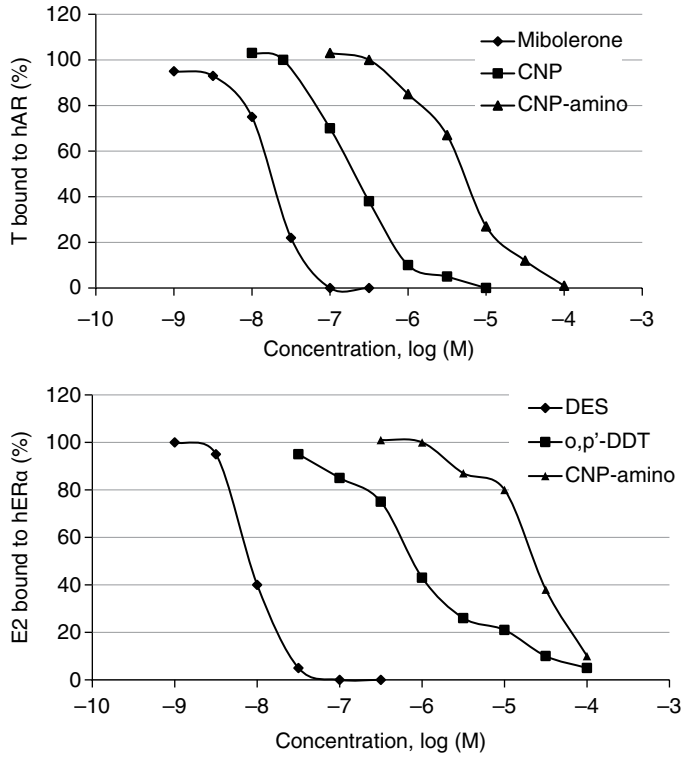


Fig. 2.31. Competitive binding assay for AR and ERα. Mibolerone is a synthetic anabolic steroid that binds AR, and DES is a synthetic oestrogen that binds ERα. CNP, chlornitrofen, 2,4,6-trichlorophenyl-4′-nitrophenyl ether; *o,p′*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (adapted from Kojima *et al*., 2003).

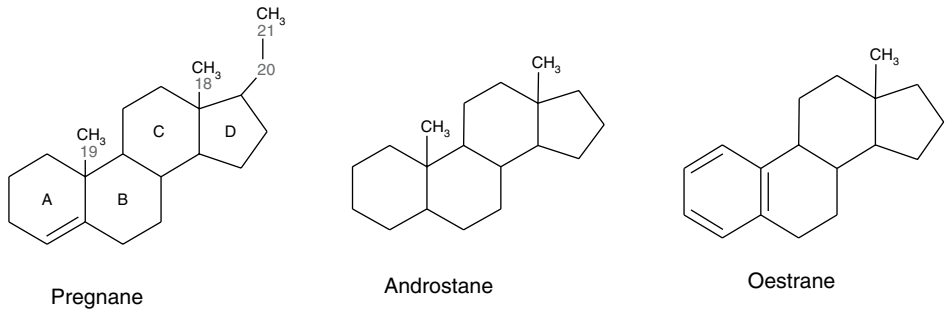


Fig. 2.32. Pregnane, androstane and oestrane steroid structures. Pregnane (C21) indicates a steroid structure with methyl groups at C13 and C10 and a two-carbon-atom side chain attached at the C17 position. Androstane (C19) indicates a steroid structure with methyl groups at both the C13 and C10 positions. Oestrane (C18) indicates a steroid structure with a methyl group attached at C13 and an aromatic A ring.

Because of this species specificity, peptide hormones must be isolated from the same or very similar species for biological activity. They have a higher molecular weight than steroids and this makes structure elucidation more difficult. Proteins are also easily degraded by cellular proteases and this can result in a number of protein fragments being produced during the isolation procedure.

Table 2.4. Systematic names of vertebrate steroids.

Class name	Trivial name	Systematic name
Glucocorticoids	Cortisol (hydrocortisone)	11β, 17α, 21-Trihydroxy-pregn-4-ene-3,20-dione
	Corticosterone	11β, 21-Dihydroxy-pregn-4-ene-3,20-dione
Mineralocorticoids	Aldosterone	11β, 21-Dihydroxy-pregn-4-ene-3,20-dione-18-al
	11-Deoxycorticosterone	21-Hydroxy-pregn-4-ene-3,20-dione
Androgenic steroids	Dehydroepiandrosterone	3β-Hydroxy-androst-5-ene-17-one
	Testosterone	17β-Hydroxy-androst-4-ene-3-one
Oestrogenic steroids	Oestradiol	1,3,4(10)-Oestratriene-3,17β-diol
Progestens	Pregnenolone	3β-Hydroxy-pregn-5-ene-20-one
	Progesterone	Pregn-4-ene-3,20-dione

This problem can be reduced by including protease inhibitors in the isolation buffers. Many protein hormones are also modified after translation, for example by glycosylation or phosphorylation, and this can be essential for hormone activity.

For interest

Historical perspectives of protein and peptide hormone purification

Insulin was crystallized by Abel in 1926, who demonstrated it was a protein. White and colleagues first crystallized prolactin in 1937. In 1953, the structures of oxytocin and vasopressin were determined and these peptides were then synthesized by du Vigneaud. Sanger determined the structure of insulin, and it was synthesized in the 1960s. Glucagon was crystallized by Staub and sequenced, in 1957, by Bromer and colleagues. See Hadley and Levine (2006).

Determination of amino acid sequence

Peptide and protein hormones consist of a linear chain of amino acids, with the sequence of the amino acids determining the primary structure of the protein. Short peptides can be sequenced directly, but long peptide chains are divided up into smaller chains by enzymatic digestion to obtain overlapping fragments, which are then sequenced. However, it is much easier to obtain the amino acid sequence by sequencing a cDNA encoding the protein, if it is available (see below).

Peptides were traditionally sequenced from the amino-end terminal by Edman degradation (Fig. 2.34). This process is now done by automated methods. The amino-end terminal amino acid of the peptide is first labelled with phenylisothiocyanate and then the labelled amino acid is removed by mild acid hydrolysis. The labelled amino acid that is released by hydrolysis is identified by chromatography. The cycle is then repeated with the shortened peptide.

Peptides and proteins can also be sequenced using matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry. This is used especially in the field of proteomics, to sequence a large number of proteins that are expressed in a variety of biological samples. This type of mass spectrometry generates overlapping short peptide fragments and determines their molecular weights. The sequence of amino acids in the peptide fragments is then deduced from these molecular weight measurements.

The amino acid sequence can also be determined indirectly from the DNA sequence and this is particularly useful for large peptides and proteins (Fig. 2.35). If the identity of the protein is known but the sequence is not known for the species of interest, it may be possible to obtain the sequence by *in silico* analysis. This involves searching online databases such as Genebank at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) using the known sequence (e.g. from human) to search for expressed sequence tags (ESTs or short cDNA reads) from the species of interest (e.g. pig) that are homologous to the known sequence. These ESTs are then aligned (for example, using the human template) to generate the desired cDNA sequence (e.g. in pig), which is then translated to give the desired amino acid sequence. To confirm that the cDNA sequence is correct, mRNA is isolated from a tissue that expresses the protein, and total cDNA is produced from the mRNA by reverse transcription. The cDNA for the protein is then amplified by PCR using specific primers designed from the sequence,

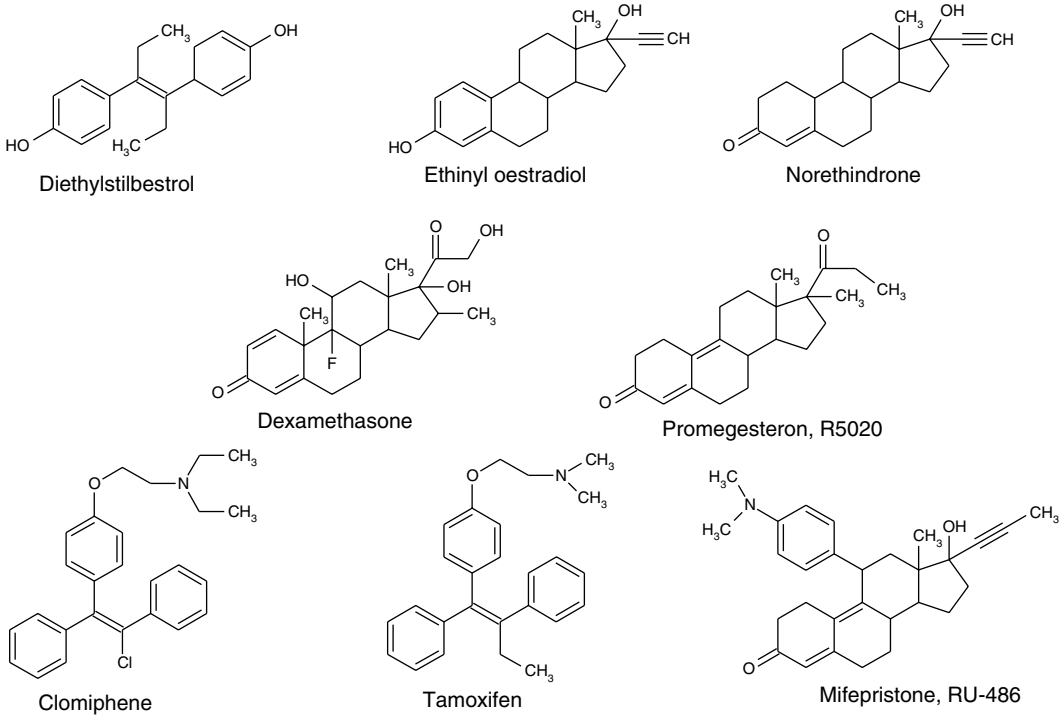


Fig. 2.33. Synthetic steroid agonists and antagonists.

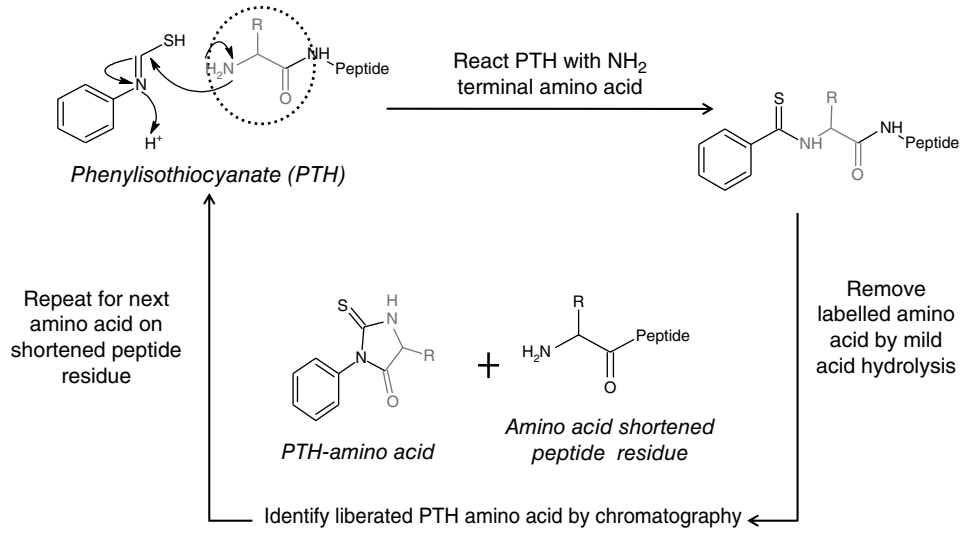


Fig. 2.34. Determination of amino acid sequence of proteins by Edman degradation.

and the PCR products are used for direct sequencing of the cDNA.

If the identity of the protein is not known, the protein is purified and a partial sequence of the protein is determined directly. This information is used to design DNA primers encoding both ends of the protein. In cases where more than one codon is used for an amino acid, degenerative primers are designed using the codon combinations that are commonly used for this amino acid. The cDNA encoding the protein is then amplified by PCR from cDNA produced from a tissue that expresses the protein. The sequence of the cDNA is determined and the amino acid sequence is deduced from it.

Peptide and protein synthesis

The chemical synthesis of peptides involves the formation of peptide bonds between amino acids that are arranged in a specific sequence. Since amino acids are at least bifunctional, with both an amino group and a carboxyl group, the groups that are not involved in the formation of peptide bonds must be protected (Fig. 2.36). The carboxyl and amino ends, as well as selective side groups not involved in the desired peptide bond, are first blocked and then the peptide bond is formed. The desired blocking group is then removed from the peptide and the next amino acid is added.

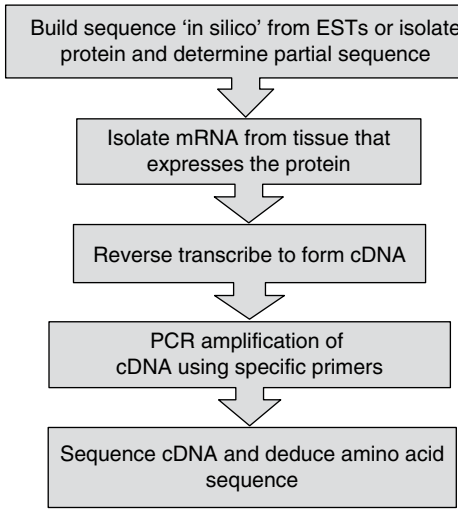


Fig. 2.35. Strategies for deducing amino acid sequence from cDNA.

The chemical synthesis of larger peptides uses a solid-phase support to assemble the peptide (solid-phase peptide synthesis (SPPS) or Merrifield synthesis) in a manner that is analogous to the function of a ribosome in the cell (Fig. 2.37). During the synthesis, the peptide remains covalently attached to polymeric resin beads, so excess reagents and side products can be removed by washing and filtration. This improves the recovery and efficiency of peptide synthesis. The disadvantages of chemical synthesis are the high cost for equipment and reagents, the limitations of peptide size (~30 amino acids) and the low recovery of the peptide from the synthesis.

Examples of peptides that have been made by solid-phase synthesis include bradykinin (9 residues), vasopressin (9 residues), valinomycin (12 residues), neurotensin (13 residues), β-endorphin (31 residues), corticotrophin (39 residues), ferredoxin (55 residues) and β-lipotrophin (91 residues).

Non-peptide mimics of peptides

A large number of biologically important peptides are known, but small peptides are limited in their usefulness as hormone supplements. Peptides suffer from poor bioavailability, low metabolic stability, lack of oral activity and rapid excretion since they are rapidly degraded by proteases and have a limited ability to penetrate membranes. To overcome these

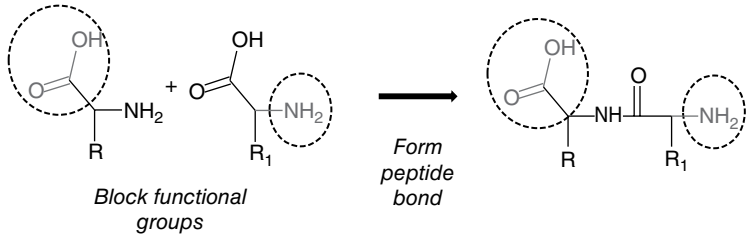


Fig. 2.36. Strategies for chemical synthesis of peptides. Functional groups that are blocked are outlined.

problems, non-peptide compounds called peptidomimetics have been developed to act as orally active agonists or antagonists for peptide hormones. These non-peptide compounds mimic the three-dimensional structure of the hormone ligand, and an unlimited variety of compounds can be made with various biological activities (for example, Fig. 2.38). Different libraries of chemical structures are used based on a scaffold core 'privileged structure' that mimics the secondary structure of the amino acid sequence that is essential for specific binding to receptors. Scaffolds based on cyclopropane have been successfully applied to many bioactive peptides. Cyclic peptides such as cyclosporine have enhanced permeability to cell membranes, and other membrane-permeable peptidomimetics have been developed. Often small alterations in the molecule, such as alkyl substituents or changes in the stereochemistry, change the biological activity from agonistic to antagonistic.

The earliest examples of these hormone analogues were based on morphine and related opioids, which mimic the biological activity of the enkephalins (natural peptide analgesics). In fact, the structure–activity relationships of the opioids were determined before their receptor was characterized. More recently, non-peptide analogues for the growth hormone secretagogues (GHS) and cholecystokinin (CCK) (see Section 3.4, Fig. 3.16; and Section 3.10, Fig. 3.37) have been developed using functional screening assays. GHS analogues were developed based on structural elements in the hexapeptide growth hormone-releasing peptide (GHRP) that were thought to be critical for

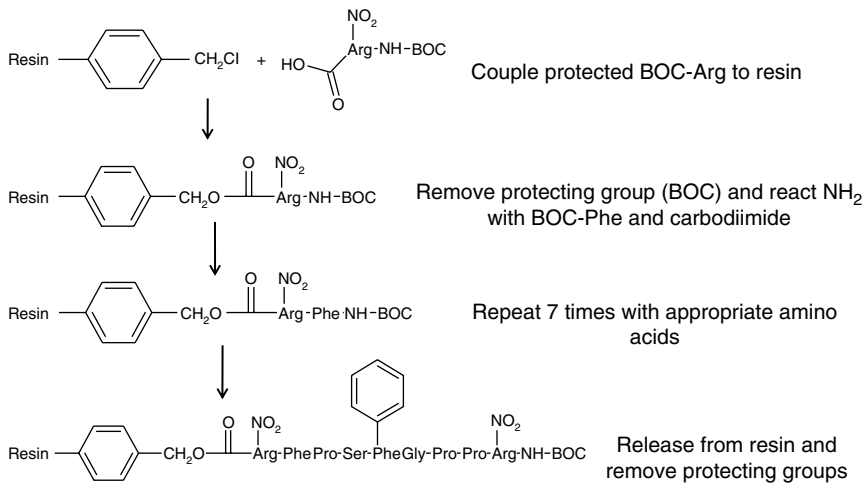


Fig. 2.37. Merrifield synthesis of bradykinin.

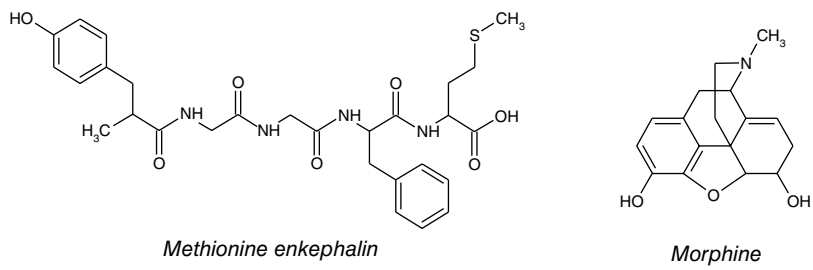


Fig. 2.38. Examples of non-peptide mimetics of hormones.

bioactivity. A large number of compounds were then screened for their effectiveness in releasing growth hormone from a rat pituitary cell bioassay. Similarly, agonists for the CCKA receptor were screened for their effectiveness in contracting a gall bladder preparation bioassay. Once biologically active compounds are found, the pharmacokinetic parameters are measured to determine the *in vivo* effectiveness of the compounds. For further information, see the review by Mizuno *et al.* (2017).

Production of recombinant proteins

An efficient method for producing large peptides or proteins is to express these proteins *in vitro*, in bacteria or eukaryotic cells, or *in vivo* using transgenic animals. For the *in vitro* production of proteins (Fig. 2.39), the DNA sequence encoding the protein is isolated as described above and inserted into an expression vector, which is transfected into bacteria or a eukaryotic cell line (see Section 2.1 for information on cell lines). As the cells grow, the expression vector directs the synthesis of large amounts of the protein, which is then purified.

There are a number of points to consider for *in vitro* production of recombinant proteins. Once the DNA sequence that codes for the protein has been isolated, it is cloned into an expression vector that is appropriate for the cell type being used to give a robust production of protein. Bacteria are easy to grow and can produce large amounts of recombinant proteins (up to 30% of their total protein) but the choice of using a bacterial or eukaryotic expression system depends on the protein to be expressed. Bacteria do not modify proteins after translation, so if there is post-translational modification of the protein, such as glycosylation that is required for activity, a eukaryotic system must be used. Bacteria also use methionine for the initial amino acid, so any protein produced in a bacterial system will begin with methionine and this can be potentially immunogenic.

Purification of recombinant proteins produced in eukaryotic cells can be simplified by adding a signal peptide to the gene, so the protein is exported from the cells. The protein is then harvested from the culture medium, which reduces the number of compounds that have to be removed during the purification.

A common method for expression of recombinant proteins is to produce proteins as fusion products to stabilize the protein, make the protein more soluble or act as convenient tags for purification by affinity chromatography. The fusion protein can then be cleaved after the purification to produce the recombinant protein. Examples of fusion proteins include β-galactosidase, bacterial alkaline phosphatase, chloramphenicol acetyl transferase, green fluorescent protein (GFP), glutathione-*S*-transferase and luciferase. Examples of smaller fusion peptides are the FLAG sequence, which contains the rare five amino acid recognition sequence for enterokinase (Asp-Asp-Asp-Asp-Lys ↓ X) and the V5-His tag. These small peptides are particularly useful as they may not affect the function, secretion or transport of the fusion protein and thus may not need to be removed, but they are useful for purification of the expressed proteins. They can also be used to detect the

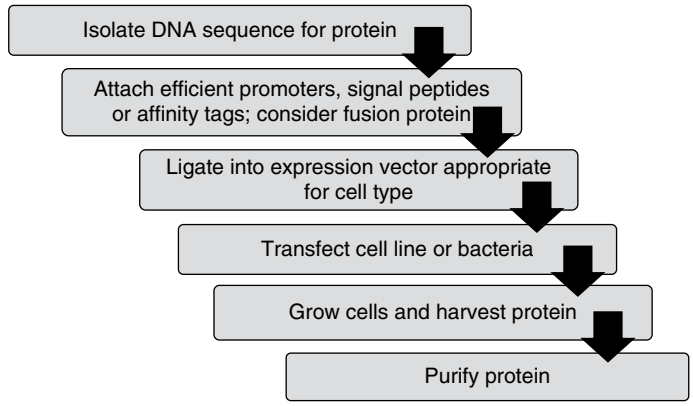


Fig. 2.39. Production of proteins *in vitro* using recombinant DNA techniques.

tagged proteins using antibodies against the tag in a Western blot or ELISA.

Recombinant protein hormones can also be produced *in vivo* using transgenic animals. This involves creating a transgenic animal that acts as a 'bioreactor' to produce the desired protein and deposits it in milk, eggs or semen so it can be easily harvested. This may be a cost-effective method overall to produce large amounts of protein but it requires considerable effort to first generate the appropriate transgenic animal that produces the recombinant product. It is important to control the expression of the transgene and this can be accomplished using tissue-specific promoters or inducible promoters. Examples of tissue-specific expression include the use of casein promoter for the production of milk protein and the use of the ovalbumin promoter to have the product deposited in the egg. For more information on the production of transgenic animals and proteins produced by them, see Section 2.4.

The advantages of recombinant DNA technology methods for producing proteins include the lower cost overall, high efficiency and the production of a higher-purity, consistent product. The disadvantages are that an appropriate DNA construct must first be produced and that simple bacterial systems cannot always be used. In some situations, the 'pro' and signal sequences must be removed from the cDNA and this may affect the folding of the polypeptide chain and reduce the activity. There is also the possibility of degradation of foreign polypeptides when they are expressed in a cell. In bacterial systems, proteins can also accumulate as insoluble form in inclusion bodies. For more information, see Rosano and Ceccarelli (2014) and Tripathi and Shrivastava (2019).

2.4 Manipulation of Endocrine Function

Key concepts

- Endocrine systems can be manipulated by altering endogenous hormone levels, administering long-acting analogues or affecting the signal transduction process.
- A hormone can be administered as a feed additive if it is not degraded by the digestive system or rapidly metabolized and eliminated.
- Hormones that are not orally active can be administered as single or multiple injections or in long-acting implants.
- Active compounds can be encapsulated to increase their efficacy, reduce potential toxicity and improve uptake by the target tissue.
- Hormones and other bioproducts can potentially be provided by an implant containing encapsulated cells that produce the product.
- Pulsatile release can potentially be obtained from implants that contain hormone encapsulated in a mixture of different microspheres.
- Antibodies can be used to repress hormone action by passive or active immunization against a hormone.
- Transgenic animals can be created using CRISPR/Cas9 to alter endocrine function or used as bioreactors, to produce a valuable protein product that is deposited in the milk, eggs, semen, etc.

Introduction

The goal of manipulating various endocrine systems in order to improve animal productivity, health and welfare can be achieved in a number of different ways. Due to the multiple levels of regulation of endocrine systems (see Table 1.6), there are many opportunities to affect the activity of an endocrine system. We can influence the synthesis and release of hormones by altering the environment, for example increasing photoperiod in chickens to stimulate egg laying or adjusting the dietary protein and energy to affect the release of a number of hormones. We can also directly administer hormones and, in particular, use long-acting analogues. We can also affect other components of the signal transduction process (see Section 1.3). We can activate the appropriate kinase or inhibit the appropriate phosphatase directly to mimic hormone action. We can also block hormone action by blocking the hormone or receptor, increasing hormone turnover, blocking the second messenger, or activating the phosphatase and blocking the kinase directly.

For interest

Look for examples of a therapeutic compound that works by inhibiting the activity of a specific protein kinase or protein phosphatase. What is the significance of treatment with this compound?

Additional ways to manipulate endocrine systems include genetic selection and production of transgenic animals. Genetic selection involves identifying polymorphisms in the genes encoding some component of the system, such as the hormone or its receptor, which affects the activity of the system. The genotype of the polymorphism within the population is determined and correlated with the trait of interest. Animals with the appropriate genotype can then be selected for breeding programmes. Producing transgenic animals involves modifying the normal function of the system by gene editing, gene insertion or gene knockout, as described above and later in this section.

For interest

Historical aspects of hormone use

The earliest use of hormones to enhance production in farm animals involved feeding iodinated proteins to dairy cows to increase milk production and using diethylstilboestrol (DES) and dienesterol in broilers for enhanced fat. In the USA, DES was used from 1954 in beef cattle and sheep to increase growth efficiency and lean yield, but its use was banned in 1972 due to potential carcinogenicity in humans. In 1958, oestradiol benzoate/testosterone implants were approved for heifers, and in 1969, zeranol implants were approved. The USA approved bovine somatotrophin (bST) for improving milk production in dairy cattle in 1993 but its use was not approved in Canada.

Hormone delivery methods

Hormones can be administered orally as feed additives if they are not degraded by the digestive system or rapidly metabolized and eliminated. This generally works for small molecules but not for unprotected proteins. Examples of these orally active compounds are β agonists (see Section 3.7) or the synthetic progestagen, melengesterol acetate (MGA) (see Section 3.2). For this application there is a potential safety concern for feed-mill workers from exposure to hormones in the dust. Methods for oral delivery of proteins have been developed based on encasing the proteins in hydrogels of casein/calcium phosphate/polyethylene glycol. Polyethylene glycol (PEG) is widely used in protein-conjugation polymer coatings to extend the half-life of particles in the circulation, decrease immunogenicity and increase solubility of proteins. This has been used to protect insulin from degradation in the stomach and deliver it to the intestine, where it can be absorbed to some extent. Enteric coated capsules that are sensitive to changes in pH can be used to release the hormone in specific locations of the gastrointestinal tract. Cell membrane permeability enhancers and pH modulators (such as lactic acid) have been used to increase the absorption of an oral glucagon-like peptide 1 (GLP-1) analogue. Small molecules with pH-dependent solubility can also be treated with pH modifiers or formulated as alkyl ester derivatives that mask ionizable groups to improve solubility.

Microspheres can be formulated into aerosols for inhalation and targeting to the lung (e.g. inhaled insulin). Permeability enhancers can also be included so that larger proteins (up to 20 kDa) can be taken up across the mucosal epithelium. Nanoparticles that are three to four orders of magnitude smaller than microspheres can be taken up by cells and used to deliver compounds to cells, for example encapsulating interleukin 12 for treatment of tumours, or for delivery of DNA and RNA vaccines. Large molecules can be moved across the blood–brain barrier by targeting transcytosis receptors, such as the transferrin receptor or insulin receptor on the blood–brain barrier. The proteins are linked to carriers called 'molecular Trojan horses', or a fusion protein that recognizes the receptor can be used. For more information on these technologies, see the review by Slastnikova *et al.* (2018).

Hormones that are not orally active can be administered as single or multiple injections, usually as subcutaneous or intramuscular injections. Multiple injections result in a pulsatile delivery of hormone, while a single dose produces high levels that decrease over a period of time (Fig. 2.40). Hormones can be given as a suspension in oils or waxes, to give a more sustained release than hormone in aqueous solution.

Encapsulating an active compound before intravenous injection can increase its efficacy and reduce potential toxicity. The compound could also be chemically modified to a derivative that is activated at the target site. Encapsulation protects the compound from degradation during transport to the site of action, thus increasing the exposure where it is needed while reducing toxicity by decreasing exposure to all other tissues. Potentially,

delivery systems can be devised to target particular cells and direct compounds to tissues where they are biologically active. Specific carriers can be utilized, such as liposomes, microspheres, nanoparticles, antibodies, cells (e.g. erythrocytes and lymphocytes) and macromolecules. The activity of therapeutic proteins can also be extended by altering the protein structure, including coupling to PEG, glycosylation or altering amino acids. For example, anti-tissue necrosis factor (TNF) antibodies have been coupled to PEG for preferential accumulation in inflamed joints after intravenous administration. Alternatively, compounds can be coupled to a hormone for delivery to the target tissue of the hormone. For example, *in vivo* treatment of ewes with a GnRH agonist coupled to a cytotoxic agent (pokeweed antiviral antigen) decreased the ability of the pituitary gland to secrete LH. This could provide a novel approach to sterilizing animals. Hecate, a conjugate of a lytic peptide, coupled to a segment of the β chain of LH selectively killed cultured cells expressing the LH receptor, including prostate cancer cells.

For interest

Find other examples of hormones that have been microencapsulated and are approved for commercial use. What are they used for? How could they be used for commercial animals?

Hormones can be delivered with sustained-release devices that are implanted in the animal, which is useful for long-lasting hormone treatment. This gives an initial rapid release of hormone, which may be above the optimal concentration, which decreases gradually (Fig. 2.40). This super-optimal dose can be a problem and potentially desensitize the system. Subsequent implants can be given as the concentration of hormone drops below the effective dose. The hormone composition of the implants could also be varied as the animal develops. Sustained-release devices have the advantage of being less labour intensive than multiple injections.

Types of sustained-release devices

Growth-promoting implants have been used extensively in cattle in North America since the early 1970s (see Table 3.1). These are implanted in the base of the ear, usually with a large-bore needle. Another example is the GnRH analogue leuprolide acetate formulated in microspheres (Lupron Depot®) that releases the hormone for up to 6 months. The matrix of implants that binds the hormone needs to be neutral (hypoallergenic) and can be composed of lactose (short-acting), cholesterol (long-acting), or a large polymer of polyethylene glycol (PEG) or poly(d,l-lactide-co-glycolide) (PLGA). Silastic tubing impregnated with the hormone can also be used and in this case the length of the tubing controls the dose. The progesterone-

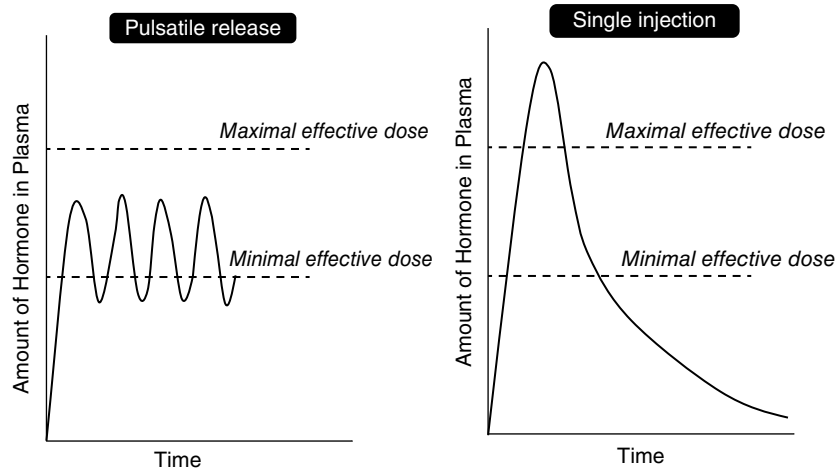


Fig. 2.40. Pulsatile release versus single injection administration of hormones.

releasing intravaginal device (PRID), which is used for synchronizing oestrus (see Section 5.1), is an example of delivering hormone to the site of action. Osmotic minipumps (Alzet®; www.alzet.com) can be used for the experimental delivery of hormones. Skin patches could also be used for uptake of the hormone through the skin.

The potential problems of using implants are that protein hormones, such as growth hormone, can form insoluble aggregates over time, so not all the hormone is released. The hormone can also degrade over time and may need to be stabilized. The implanting technique can also cause abscesses and expelled or crushed implants, or there may be infection or an immune response to the implant. To prevent this, sterile equipment should be used and the matrix should be hypoallergenic and readily release the hormone from the implant.

The use of biodegradable implants for hormone delivery that would not have to be removed at the end of the treatment period would give significant cost and labour savings and would decrease animal stress. These implants are made from biodegradable polyesters based on polylactic acid/glycolytic acid and related polymers, which have low immunogenicity and toxicity. These types of polymers are also used as biodegradable scaffolds for tissue engineering (e.g. for stimulating bone growth). In formulating implants, the components of the polymer are adjusted and different emulsifying and extraction techniques are used to affect the size, stability and porosity of the microspheres and nanoparticles that encapsulate the hormone. This controls the rate of degradation of the implant and hormone delivery. Potentially, implants can also be formed *in situ* by injecting a solution of polymer and active compound in an acceptable organic solvent. Dilution into the aqueous environment or exposure to body temperature precipitates the polymer and encapsulates the active compound to form the implant.

A novel approach to providing hormones and other bioproducts is the use of encapsulated cells. Cells that have been modified to produce a compound of interest are placed in semi-permeable microcapsules and implanted in the animal. Natural hydrogels of alginate, agarose, chitosan, cellulose, collagen and xanthan and several synthetic polymers such as PEG are used for cellular encapsulation. Several different techniques such as electrostatic spraying, emulsion, micro-nozzle array, interfacial polymerization and extrusion methods have been used to encapsulate cells. This allows for the bidirectional exchange of nutrients, oxygen, metabolites and by-products with the encapsulated cells but excludes most components of the immune system and decreases the rejection rate of the implants (Fig. 2.41 and Table 2.5). The cells can also respond to signals from the animal in real time and release the desired amount of the hormone accordingly. This approach was explored by implanting pigs with transgenic growth hormone-secreting cells encapsulated in a matrix and using encapsulated pancreatic islets for treatment of diabetes. However, there has not yet been a licensed product based on cell encapsulation technology available, with the major limitation being the host immune response against both the implanted capsule and the encapsulated

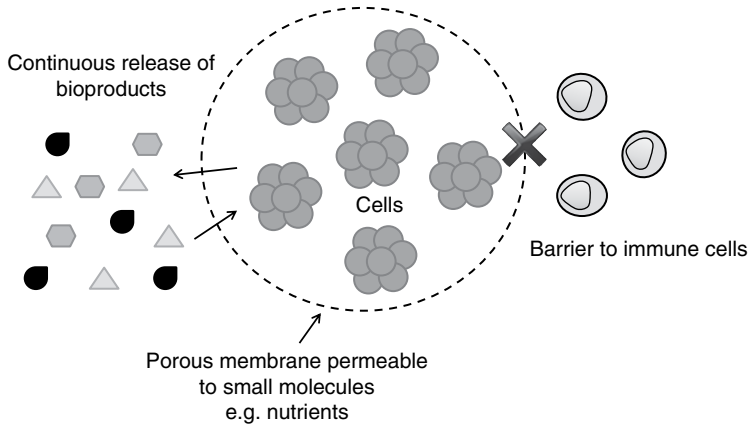


Fig. 2.41. Microencapsulation of cells.

Table 2.5. Problems associated with encapsulated cells and potential solutions (adapted from Hauser *et al*., 2004).

Issue/decision to be made	Problem/requirements	Solution
Quality of starting materials	Possible contamination with pyrogens, endotoxins, heavy metals, etc.	Use well-defined starting materials
Encapsulation	Reproducibility of process	Use well-defined parameters and optimized procedures
Cell type to be used	Cells must be well characterized, free of contaminants	Primary cells can be animal specific, but cell lines are easier to characterize and safety test
	Potential retrovirus problems and more vigorous immune response with xenogenic cells than allogenic cells	Cells should be prescreened to eliminate virus production. Include fail-safe 'suicide gene' in allogenic cells
Immune reactions	Directed towards cells	Allogenic cells are better than xenogenic cells
	Directed towards therapeutic product	Use immunosuppression
	Directed towards capsule material	Use contaminant-free and hypoallergenic material
Site of implantation	Need to provide material to required areas of the body	Implant in optimal location or multiple sites
Cryopreservation	Required for long-term storage but must maintain cell viability and capsule structure	Use cryoprotectants with controlled cooling rates

cells. For more information, see the reviews by de Vos *et al*. (2014), Esfahani *et al*. (2017) and Ashimova *et al*. (2019).

Pulsatile and controlled release of hormone

The pulsatile versus continuous mode of administration can affect hormone response. Some hormones, such as insulin, somatostatin, GH, PTH, GnRH and LH, are secreted in pulses and pulsatile delivery of these hormones gives the optimum biological effect (see Section 1.4). This may prevent the suppression of natural hormone secretion from continuous administration of large doses of hormone. Receptor numbers can also be down-regulated by negative feedback or the activity of the receptor can be decreased by continuous high concentrations of hormone (see Section 1.3).

Pulsatile release can be obtained from implants. Implanted mechanical pumps can be used but these are expensive, may require surgery and are used mainly in humans. A number of microfabricated delivery systems, such as microneedle syringes, patches, micropumps and implantable drug-releasing microchips, have been developed. These systems can release individual doses of several substances from different reservoirs. For example, an implantable multi-reservoir device for on-demand and pulsatile delivery of growth hormone has been developed (Lee *et al*., 2019).

Polymer-based hormone-releasing implants have been developed for pulsatile hormone release. Bulk eroding systems (Fig. 2.42) consist of a mixture of microspheres made of different formulations of polylactic acid/glycolytic acid and related polymers, which degrade to release hormone at different rates. Surface eroding systems (Fig. 2.43) composed of poly(ortho)ester and polyanhydride matrices degrade rapidly from the surface. More than one matrix composition can be used in layers (with hormone and then without hormone) to get pulsatile release of hormone. The composition of the surface layers can be varied to produce a mixture of microspheres that degrade at specified rates to produce a series of pulses of hormone release.

In osmotically controlled systems (Fig. 2.44), the outside of the implant is coated with a water-insoluble polymer with a water-soluble pore former; the interior contains hormone and the osmotic agent. Pores are created to allow water to move inside by osmotic pressure to rupture the implant and release a pulse of hormone.

A number of triggered pulsatile delivery systems have been developed. Enzymatically activated liposomes can be prepared containing phospholipase A2 with hormone on the inside and the liposome encapsulated in alginate-polylysine. Once the

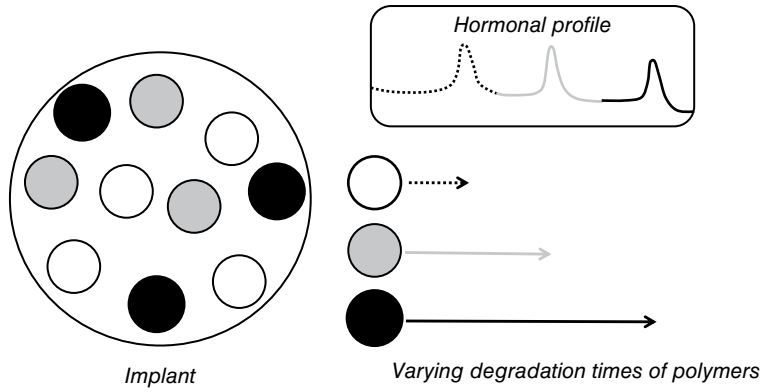


Fig. 2.42. Bulk eroding system for pulsatile release of hormones.

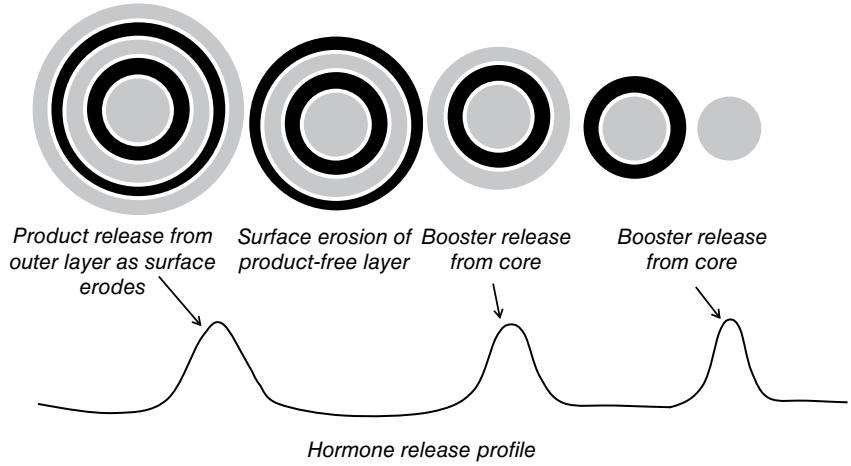


Fig. 2.43. Surface eroding system for pulsatile release of hormones.

capsule dissolves, the phospholipase degrades the liposomes to release the hormone in a pulse. Hormones can also be encapsulated in a system that releases the hormone on exposure to a chemical trigger, for example liposomes with membrane receptors that are associated with pores that open on exposure to a drug. Systems can also be designed to release hormone by exposure to changes in pH or temperature, electric pulses or magnetic fields. For more information, see Patil *et al*. (2013).

Implantable delivery systems have also been developed based on micro- and nanotechnology. These use aluminum, titanium and silicon that has been anodized and etched to produce a precisely engineered nanoporous structure for the controlled release of hormones. These have superior mechanical, chemical and thermal stability, resistance to erosion and/or biodegradability, and can be chemically coupled with different functional molecules (e.g. silanes, proteins, lipids, etc.) to improve biocompatibility. Combinations of carbon-based nanomaterials, especially carbon nanotubes (CNTs) and graphene oxide (GO) have been used with polymer-based materials such as hydrogels, nanofibres and polymer films for hormone-releasing applications. For more information on these technologies, see the review by Santos *et al*. (2014). For more information on controlled release of hormones for contraception, see the review by Sivasankaran and Jonnalagadda (2021).

Hormone residues

Hormone residues in edible tissues are a potential concern when exogenous hormones are used. For humans, the acceptable daily intake (ADI) per unit of body weight is the dose that gives no hormone effect in the most sensitive animal model divided by 100. The potential daily intake (PDI) is the average intake of animal tissue times the residue level. The PDI must be less than the ADI for approval of the hormone treatment protocol. See Section 3.2 for a discussion of safety issues related to anabolic steroids.

Usually, physiological concentrations of hormone are desired when hormones are given. Higher (pharmacological) doses may be detrimental and act through negative feedback to reduce the effect of the hormone. There are also differences between individuals and time of day effects on hormone activity. The effective dose of hormone depends on how rapidly the hormone is inactivated, so differences in metabolism among different animals may affect the extent of hormone activity. The time-of-day effects are controlled by the circadian rhythm (see Section 1.4), which regulates a wide range of biological process, including metabolism and endogenous release of hormones.

Immunomodulation of hormone action

The production of antibodies and their use in hormone assays is discussed in Section 2.1. Antibodies can also be generated to enhance or repress hormone action. Antibodies are immunoglobulins (Igs) that are present in the γ-globulin fraction of plasma proteins. They comprise two light polypeptide chains (23 kDa) and two heavy polypeptide chains (50–70 kDa). Disulfide bonds join the chains into a 'Y' configuration, with the variable (V) regions located at the distal ends of the 'Y'. The hyper-variable regions in the V regions have binding sites that are specific for particular antigens; these sites are called idiotypes. The constant (C) regions next to the variable regions are distinctive for each Ig type (Fig. 2.45).

IgM is the first antibody type formed after primary immunization and it acts to protect the intravascular space from disease. IgG is formed after IgM levels decrease and in the secondary immune response. It is the most prevalent serum antibody and is also found in the extravascular space. IgA is found in mucous secretions and is an early antibacterial and antiviral defence. IgE is found in respiratory and gastrointestinal tract mucous secretions and may be involved in the allergic response.

The immunoneutralization of hormone action can be achieved by active or passive immunization and some applications for the immunoneutralization of hormone action are summarized in Table 2.6. For active immunization, antibodies are generated within the animal by immunizing with the hormone (antigen), and the antibodies then bind to the hormone to block its effects. Some animals may not respond to the immunization procedure and generate high antibody titres, so the effect of the hormone may not be reduced. For passive immunization, purified antibodies against the hormone or the receptor are infused into the animal. This requires a source of purified antibody, which increases the cost of the procedure, but since it does not depend on an immune response from the animal, it should give consistent results. Passive immunization is thus more suitable for short-term reversible immunization but it can give more specific targeted effects if monoclonal or recombinant antibodies are used (see Section 2.1).

Antibodies can also be raised against idiotypes in another antibody, and these are called anti-idiotypic antibodies. Anti-idiotypic antibodies exist naturally and these are important in the regulation of B cell

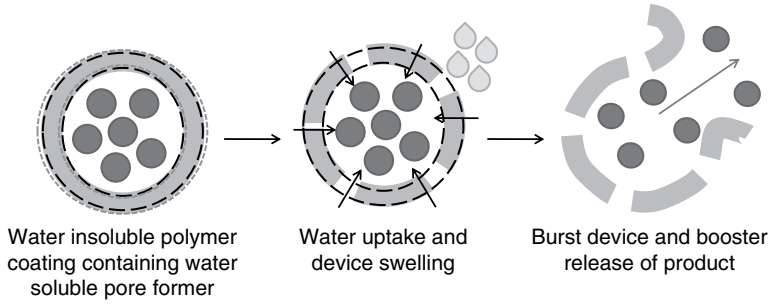


Fig. 2.44. Osmotically controlled system for pulsatile release of hormones.

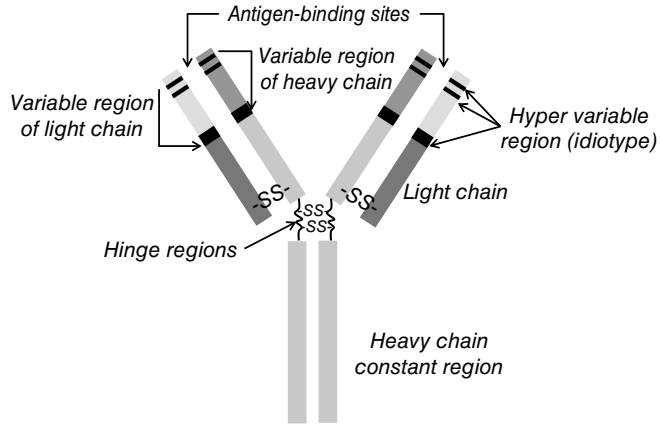


Fig. 2.45. Structure of antibodies.

Table 2.6. Applications of immunoneutralization of hormone action.

Production parameter	Immunogen	Mode of action
Increased fertility of sheep	Oestrogen – 'fecundin'	Decreased feedback from oestradiol → increased LH → increased ovulation
	Inhibin	Increased FSH → increased ovulation
Reduced libido – bulls Contraception – wildlife Immunocastration – pigs	GnRH	Decreased GnRH → decreased gonadal function
Increased growth and carcass lean	Somatostatin	Increased GH due to decreased somatostatin
Manipulation of oestrus in horses	Melatonin	Remove inhibition of reproductive activity in seasonal breeders
Decreased carcass fat	Adipocyte membranes	Decreased development of adipose tissue
Reduced boar taint in uncastrated male pigs	Androstenone	Binding of Ab to androstenone did not decrease accumulation in fat

function. Secondary antibodies that are directed against primary antibodies to a hormone can also be produced. Some of these secondary antibodies will be specific for the idiotypic region that recognizes the receptor and these can have hormonal activity. The hormonal activity from anti-idiotypic antibodies made by active immunization will be maintained longer than a hormone implant and may be longer acting and more stable than hormone treatment. There is also the added advantage of no hormone residues in the edible tissues from this treatment. The disadvantage of this procedure is that it is necessary first to generate, test and purify a primary antibody, which is then used to generate the secondary anti-idiotypic antibody. The anti-idiotypic antibody also may not have hormonal activity. Examples of anti-idiotypic antibodies that have been studied include: anti-β agonist anti-idiotypic antibody, which can act as β agonists to improve growth and carcass lean; and anti-GH anti-idiotypic antibody, to stimulate milk production in dairy cattle. Anti-idiotypic antibodies can also be tagged with a label (enzyme, chemiluminescent or fluorogenic) and used in non-competitive immunoassays. For more information, see the review by Kohler *et al.* (2019). See Erlanger and Cleveland (1992) for a method for producing monoclonal auto-anti-idiotypic antibodies.

Transgenic animals

Uses for transgenic animals

A transgenic animal is an animal that contains exogenous genes or gene modifications. These genes can be from different species and novel gene constructs can be used. Transgenic animals have applications

in improving production agriculture, as model systems to understand gene function and disease modelling and as bioreactors for production of valuable protein products (for example, in milk).

New genes can be used to modify hormone responses and thus affect the performance, growth or health of the animal. Genes for improved growth include growth hormones (GHs) and the insulin-like growth factors (IGFs). Improved productivity could also come from genes that allow better utilization of feeds or alternative feeds. Disease-resistance genes could be used to improve the health and performance of animals. For example, transgenic cows expressing lysostaphin in the mammary gland are resistant to mastitis. Altering the function of key genes can also improve health and performance. This includes resistance to porcine reproductive and respiratory syndrome (PRRS) virus-resistant CD163 knockout pigs, myostatin knockout sheep, goats and cattle with increased lean muscle yield and FGF5 knockout sheep with increased wool length and yield.

Transgenic animals can be used as model systems to study hormone function and disease modelling. This includes molecular aspects of hormone/gene interaction and *cis*- and *trans*-acting factors involved in gene regulation *in vivo*. They can also be used to identify genes important in development, to identify factors responsible for tissue-specific gene expression and to study genes involved with diseases important to both human and veterinary medicine. Livestock, especially pigs, share a similar size and physiology to humans and this makes them a good model for study of potential disease therapies prior to undergoing costly clinical trials.

Transgenic animals can also be used as bioreactors, to produce a valuable protein product that is deposited in the milk, eggs, semen, etc. (see Section 2.3). This is done by using, for example, a mammary gland-specific promoter to drive the expression of a protein of interest, which is then deposited in the milk. Species such as mice, rabbits, pigs, goats and cattle have all been utilized to produce therapeutic proteins in milk. This allows the custom synthesis of valuable products such as protein hormones, antibodies and vaccines. There have been three approvals for therapeutic human proteins produced by transgenic animals. These include: goats producing antithrombin-III, approved to treat hereditary antithrombin deficiency; rabbits producing C1 esterase inhibitor, approved to treat hereditary angioedema; and chickens producing lysosomal acid lipase A in their eggs, approved for the treatment of lysosomal acid lipase deficiency. One interesting application was the production of spider-silk proteins in the milk of transgenic goats, but this company (Nexia Biotechnologies) ended in 2009. For more information on transgenic livestock, see the reviews by Van Eenennaam *et al*. (2021), Long (2014) and Wheeler (2013).

Production of transgenic animals

A transgenic animal is produced by inserting a new gene or genes, or by modifying the expression of existing genes. Interrupting the expression of particular genes can make gene 'knockouts' and these are useful in determining the physiological role of particular gene products. Specific genes can be targeted by the use of gene constructs that are highly homologous to the target gene but contain a mutation that affects the function of the gene. The mutation is introduced into the target gene by the process of homologous recombination.

The expression of a particular gene can also be reduced by RNA interference-mediated gene knockdown (RNAi). RNAi is a conserved post-transcriptional process in gene regulation and in the innate defence against invading viruses that silences gene expression by promoting the degradation of mRNA. It involves short double-stranded small interfering RNA (siRNA) and microRNAs (miRNAs) which bind to complementary sequences in mRNA. SiRNA binds to complementary sequences in a specific mRNA to cause endonucleolytic cleavage of that mRNA, while miRNA binds to 3′ sequences in multiple mRNAs to decrease their translation. Synthetic siRNAs and miRNA can be transfected into cells or infused into an animal for transient gene knockdown, or a transgenic animal that expresses the siRNA or miRNA of interest can be created for permanent gene knockdown. For more information, see Lam *et al*. (2015).

Creating a transgenic animal by inserting a new gene requires a gene to transfer, an efficient method to transfer the gene and an appropriate cell type to receive the transgene. The gene of interest is first identified and isolated. It is then linked to appropriate control elements so that it is expressed in the appropriate cell type in the animal and at the right time in development. These control elements can be found in other genes that are expressed in the tissue

of interest. Gene constructs can be made by linking the control region of interest to a marker gene that is easy to measure (Table 2.7). The expression of these test genes can be studied first in tissue culture (see Section 1.3 on gene reporter assays). A transgenic animal is then made to determine the physiological significance of expression of particular genes and how hormones control the genes.

A gene transfer methodology is used to transfect the cell type of choice. In order to create a transgenic animal that passes the transgene to its offspring, the transgene must be inserted into the germ line. Transfection of somatic cells will not produce transgenic offspring but can be used for transient gene expression in an existing animal. The use of embryonic stem cells allows the precise deletion or insertion of genes in the genome via homologous recombination (gene targeting) and then the modified stem cells are injected into a blastocyst and implanted in a recipient female. However, recombination events are rare and thus require a selectable marker (such as an antibiotic resistance gene, Table 2.7) for them to be identified. Transformation of adult/differentiated cells with a gene construct followed by nuclear transfer to an enucleated oocyte also allows targeted gene transfer. In addition, this method can be used to produce 'clones' of valuable animals.

Gene transfer techniques have evolved from non-specific insertion of gene sequences, to targeted delivery and editing of specific sequences in the genome. Pro-nuclear injection has been used to introduce a DNA construct into a fertilized egg, which is then transferred to a recipient female. Sperm-mediated gene transfer involves incorporating the gene construct into sperm, which are then used to fertilize an egg. Both of these techniques result in random insertion of the construct, with no control over the location or copy number of the transgene. For more information on how gene transfer technologies have changed over time, see Yum *et al*. (2018).

Table 2.7. Marker genes used for gene function studies.

GFP	Green fluorescent protein
β-GAL	β-Galactosidase
CAT	Chloramphenicol acetyltransferase
ALP	Alkaline phosphatase
ADH	Alcohol dehydrogenase from *Drosophila*
LUC	Luciferase
NEO	Resistance to antibiotic G418
HPRT	Resistance to methotrexate

We now have genome editing techniques where DNA is inserted, replaced, or removed from a genome using artificially engineered nucleases, or 'molecular scissors' that create double-strand breaks at desired locations in the genome. The earlier forms of sequence-specific nucleases for gene editing include the zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs comprise zinc finger proteins linked to a nuclease domain. The zinc finger sequences target a specific DNA sequence on genomic DNA which is then cleaved by the nuclease. TALENs also recognize specific sites on DNA and comprise modular DNA recognition elements found in plant pathogens fused to a nuclease domain. However, the widespread use of ZFNs and TALENs has been limited by difficulties of protein design, synthesis and validation, since targeting a new DNA sequence of interest requires the engineering of a new protein that recognizes that new DNA sequence.

The CRISPR/Cas9 method is currently the method of choice for genome editing (Fig. 2.46) and the Nobel Prize in Chemistry 2020 was awarded to Emmanuelle Charpentier and Jennifer A. Doudna for the development of this method. CRISPR is an abbreviation for 'clustered regularly interspaced short palindromic repeats', which is an array of repeat sequences in bacteria with intervening protospacer sequences that are derived from DNA from previous infections by bacteriophages and plasmids, along with an adjacent set of genes encoding CRISP- associated (Cas) nuclease.

The CRISPR/Cas system functions as an adaptive immune response of bacteria to combat further attacks by viruses. When a new virus infects the bacteria, Cas9 cuts out a segment of the virus, which is added to the CRISPR array between the CRISPR sequence repeats. The CRISPRs are transcribed into long RNA molecules (pre-crRNA) by the bacteria, which are then cleaved within the repeat sequences to yield small CRISPR-RNAs (crRNAs). This pre-crRNA processing occurs by base pairing of a trans-encoded small RNA (tracrRNA) with the repeat regions of the CRISPR and subsequent processing by the enzyme RNase III. The crRNAs bind to one or more Cas proteins to form an effector complex that targets the protospacer sequences in the invading plasmid or virus DNA. A short protospacer-adjacent motif (PAM) sequence is located about

2–6 nucleotides downstream of the target sequence and is required for the Cas9 complex to bind and cleave the target protospacer sequence. The Cas9 protein contains two nuclease domains, HNH and RuvC, that each cleaves one strand of the target double-stranded DNA.

Charpentier and Doudna demonstrated that the RNA components (crRNA and tracrRNA) of the Cas9 complex could be fused together to form an active, chimeric single-guide RNA molecule (sgRNA). The 'seed' sequence of the chimeric sgRNA could be changed to target Cas9 to the DNA sequences of interest. The Cas9/sgRNA complex first locates the PAM sequence and then cleaves the DNA at the nearby target site. Thus, Charpentier and Doudna created a simple two-component endonuclease system, containing sgRNA and Cas9, which could be programmed to cleave specific DNA sequences at will, with the only constraint being the presence of a PAM sequence adjacent to the targeted DNA. Fortunately, there is a wide variety of Cas9 proteins available that recognize different PAM sequences, so most areas of the genome can be targeted.

The double-strand breaks created by the Cas9 nucleases are repaired either by non-homologous end-joining (NHEJ) to disrupt a gene and create gene 'knockouts' or homology-directed repair (HDR) to allow gene correction or insertion (Fig 2.47).

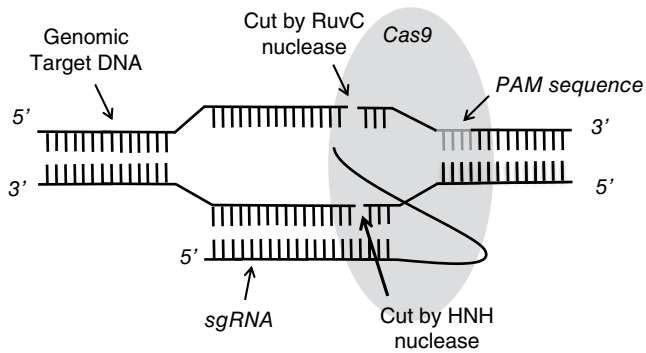


Fig. 2.46. The CRISPR/Cas system for gene editing. Site-specific DNA cleavage of the genomic target sequence by nuclease Cas9 is directed by a single-guide RNA (sgRNA) when a protospacer-adjacent motif (PAM) is present on the opposite strand.

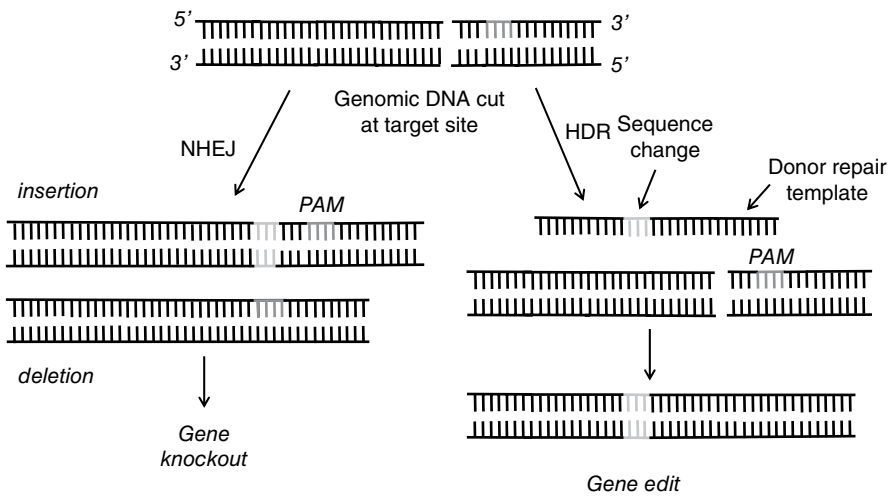


Fig. 2.47. Double-strand breaks are repaired either by non-homologous end-joining (NHEJ) to produce insertion or deletion mutations or by homology-directed repair (HDR) with a donor template to make a gene edit.

With NHEJ, the ends are directly ligated back together, and this usually results in a small insertion or deletion of DNA at the break which disrupts gene expression. With HDR, a homologous DNA sequence is used as a template to repair the break. A defined genomic change such as base substitutions for gene corrections, or insertions to add a new gene sequence, is made using a modified gene sequence as a template to guide the HDR. The PAM sequence is not included in the repair template to guide HDR, which prevents the repaired area from being cleaved again by Cas9 cleavage. This is done by altering the PAM sequence in the HDR template with a silent mutation that does not change the amino acid sequence.

Single-base editing can be done with a modified Cas9 without creating double-strand breaks and this reduces the frequency of errors that occur during the repair process. This system uses a special guide RNA containing the desired base edit that directs Cas9 with nickase activity (that cuts only one strand of the DNA) and a reverse transcriptase (RT) enzyme to the target site. There, the RT enzyme makes a new DNA strand from the RNA template and inserts it at the cut target site.

The CRISPR/Cas system has the advantage that the DNA recognition depends not on the protein, as in ZFN and TALEN, but rather on the sgRNA, which has a complementary sequence to the target DNA. It is technically much easier to generate specific RNA sequences than to engineer proteins with a defined sequence. For further details on the mechanism of CRISPR/Cas9, see the reviews by Jiang and Doudna (2017) and Liu and Doudna (2020).

The CRISPR/Cas9 system can also be used to target a gene of interest and alter its expression without cutting the DNA backbone, by using a deactivated Cas protein bound to activator or repressor domains. This method can be used to alter the transcription of gene(s) of interest in either CRISPR activation (CRISPRa) or loss-of-function CRISPR interference (CRISPRi) experiments. This allows for up-regulation or repression of a target gene in its native state without the possibility of DNA damage from off-target site effects of CRISPR.

Another approach to introducing foreign genes into an animal is by so-called gene therapy. This involves introduction of a plasmid containing the gene of interest into somatic cells, usually muscle, by electroporation. For example, pigs injected intramuscularly with a plasmid which expresses GHRH followed by electroporation had increased GHRH released into the systemic circulation, which increased the release of GH and IGF-1. This increased weight gain and lean body mass in growing pigs, while in gestating sows this improved piglet health and growth performance. While the plasmid expressed GHRH for a considerable time, it is expressed in somatic cells and is not passed on to the offspring.

Questions for Study and Discussion

Section 2.1 Methods for studying endocrine function

1. Using an *in vivo* model, how would you identify or prove the existence of an endocrine tissue?
2. Describe three methods for inactivating endocrine tissues and give an example for each method.
3. What are the advantages and limitations of *in vivo* and *in vitro* models? Discuss how you would decide on the appropriate experimental model to use.
4. Search the recent scientific literature to find papers that use two different model systems to study a particular problem in endocrinology. Discuss if the models used were appropriate to achieve the objectives of the work. How were the different studies related?
5. What model (technique) would you use to analyse the effect of hormones on activation of an intracellular enzyme?
6. A new molecule is suspected of being a hormone acting on feeding behaviour. What would you do to show it is a hormone?
7. Briefly describe how perfused organ culture works. How could the metabolites produced by the perfused organ be identified based on their chemical/physical properties?
8. What is a cell line? What are the advantages and disadvantages of using a cell line versus primary tissue culture?
9. What are important factors to consider in the culture of primary cells?
10. Describe the perifusion system of cell culture. What are the advantages of this system?
11. Describe the culture of cells to form organoids and organ-on-a-chip. How is this used in the modelling of cell–cell interactions?
12. What is an agonist? What is an antagonist? How are they useful for the study of endocrine systems?

13. How are polyclonal antibodies raised and how can you make a hormone more antigenic? How can antibodies be purified and why is this important?
14. Describe the process of producing monoclonal antibodies.
15. Describe how recombinant antibodies and bispecific antibodies are produced.
16. What are aptamers and how are they produced? Why are they an attractive alternative to antibodies?
17. Describe applications for using antibodies

Section 2.2 Measurement of hormones and receptors

1. What are the steps for validation of assay methods? Why is 'parallelism' of standard curves important?
2. What is the principle behind a biological assay (bioassay)? What are the advantages and limitations of bioassays? Why is it important? What additional information does a biological assay give compared with an immunoassay? Why is it considered the 'gold standard'?
3. Describe chemical assays. What are the advantages and disadvantages of chemical assays? Describe the different types of chromatography for analytical use.
4. How can you determine the chemical composition of a hormone (steroid versus protein)? What methods are useful for purifying and measuring protein versus steroid hormones?
5. Describe the principle and methods of competitive binding assays for the measurement of hormones.
6. You are developing a new immunoassay. Describe three steps you would take to validate your assay methods. Briefly describe how to set up a standard curve for a competitive immunoassay. After establishing the standard curve, what steps would you need to take to determine the concentration of a hormone in an unknown sample?
7. Describe Scatchard analysis of hormone–receptor binding. What important information does it give? How does it differ from competitive binding immunoassays? How does cooperativity of hormone–receptor binding affect the Scatchard plot?
8. Why is the sandwich ELISA so named? Why is it more effective when contaminants are present in the sample? How does the assay response of the 'sandwich' or 'trap' ELISA change as the concentration of unlabelled hormone increases? What key characteristic of the standard curve reflects this relationship?
9. How are the slopes of the standard curve different between a competitive immunoassay and a sandwich ELISA? Explain why the slopes differ.
10. You have just discovered a new hormone with a highly positively charged structure. Unfortunately, no antibody against it is available and its receptor is unknown. However, you really need to purify and quantify it. What technique would you use?
11. Explain how you determine total binding, non-specific binding and specific binding for receptor binding assays.
12. What is the principle behind displacement (competitive binding) assays for hormone–receptor binding? How can they be used?

Section 2.3 Methods for the production of hormones

1. What are the sources of steroid hormones? Describe the structure of steroid hormones and their synthetic analogues.
2. How is the sequence of short peptide hormones determined and how can they be synthesized?
3. What is the relative purity of steroid versus protein hormone preparations? What is the relative purity of hormone preparations from recombinant and natural sources?
4. What can be done during *in vitro* production of proteins to allow for affinity purification? Explain how affinity chromatography works.
5. Describe mechanisms whereby synthetic hormones can be more potent than natural-source hormones.
6. Outline strategies to determine the amino acid sequence of proteins by recombinant DNA methods.
7. What are peptidomimetics or peptoides and what are their advantages compared with peptide hormones? What is a 'privileged structure' and how is it used to design peptidomimetics?
8. Outline strategies for the synthesis of protein hormones using recombinant DNA.

Section 2.4 Manipulation of endocrine function

1. Discuss the advantages and disadvantages of different methods for delivering hormones.
2. Briefly describe the pattern of hormone release from sustained hormone release devices and give an

example of how they are used in animal production systems. Explain one issue with the use of sustained release devices.

3. Describe potential approaches to produce pulsatile hormone release from implants.

4. Describe the potential use of encapsulated cells for hormone replacement.

5. Outline the method and application of immunoneutralization of hormones.

6. Discuss the use of anti-idiotypic antibodies to mimic hormone action.

7. Describe the CRISPR/Cas9 system for gene editing. How can transgenic animals be made and how can they be used to affect endocrine function? What are two main applications of transgenic animals?

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3 Manipulation of Growth and Carcass Composition

This chapter covers the endocrine systems that affect growth and carcass composition of livestock. The overall endocrine effects on growth, feed efficiency and lean yield and the effects on the development of muscle, adipose tissue and bone are first summarized. The hormones affecting growth are then covered, starting with anabolic steroids and then discussing issues related to castration of pigs and removal of these anabolic hormones. The effects of somatotrophin, adipokines, thyroid hormones, β-adrenergic agonists, dietary chromium and insulin, and dietary PUFA on growth rate, feed efficiency and carcass composition are then covered. This is followed by a discussion of the many factors that affect appetite and the role of the gut microbiome in the health and performance of animals. Finally, the endocrine response to stress and its effects on meat quality are presented.

3.1 Overview

Growth is an increase in body weight and size due to an increase in the number of cells (hyperplasia) or an increase in the size of cells (hypertrophy). Growth can occur from increased deposition of protein, measured as an increase in nitrogen retention in the carcass, as well as increased lipid deposition. Protein deposition is regulated by the balance between protein synthesis and degradation; lipid deposition is regulated by the balance between lipogenesis and lipolysis. Hormones can differentially affect the extent of deposition of muscle and fat, which affects the lean yield of the carcass. Hormones can also affect feed intake, feed conversion efficiency (the feed:gain ratio), growth performance (the rate of gain) and dressing percentage (the carcass weight:live weight ratio) (Fig. 3.1). In addition, hormones can affect various aspects of meat quality, including tenderness, juiciness, flavour, pH, colour and water-holding capacity. These hormones are discussed in detail in the following sections of this chapter.

Many of these factors are interrelated and it is important not to disregard meat quality, for example, when manipulating hormones to improve growth and feed efficiency. Similarly, it is important to stimulate sufficient bone growth to maintain bone integrity and carrying capacity of the increased skeletal mass. Skeletal muscle accounts for a large portion of resting metabolic rate and energy consumption. The increase in knowledge of hormonal effects on various aspects of growth has led to strategies for increasing the lean content in carcasses and improving feed efficiency and growth rate. Improved feed efficiency means that more meat is produced from less feed, which can lower the cost of production and reduce the amount of land necessary for grazing animals. More efficient utilization of feed also means that a lower amount of waste products, such as nitrogen, will be produced in the urine and faeces, thus reducing the environmental impact of intensive production systems. However, public perceptions that exogenously administered hormones are bad, regardless of the scientific data available, can make the commercial application of these compounds difficult.

Effects on growth, feed efficiency and lean yield

Many hormones, including growth hormone, thyroid hormones, catecholamines, insulin, glucocorticoids and sex steroids, affect growth and the metabolic processes involved in the synthesis and degradation of body tissues. The result is a shift in metabolism to direct more nutrients into muscle, with fewer nutrients either deposited into fat or excreted. In contrast to the short-term regulation of metabolism for the maintenance of homeostasis, endocrine regulation of growth involves long-term endocrine changes that drive metabolism. The hormonal regulation of metabolism is affected by nutrition and by the normal cycles of endogenous hormone secretion and sensitivity in the animal. It

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is important to consider these factors to maximize the response of animals to exogenously administered hormones. For further information, see Zhao *et al*. (2019).

The young, growing animal produces mostly muscle mass with little fat, but as the animal continues to grow, the body fat content increases. Thus, the particular endocrine systems affecting growth depend on the species, sex and stage of maturity of the animal. Treatment with growth modifiers to increase protein deposition is less effective in young animals, when the inherent capacity and efficiency of protein deposition is greater, than in older animals that are closer to their mature body size.

Protein synthesis and deposition are increased by the intake of dietary protein or limiting amino acids, such as lysine, in the growing pig. When energy is not limiting, there is a linear response of protein synthesis to intake of dietary protein, or the first limiting amino acid, up to a plateau that is determined by the inherent capacity of the animal for protein deposition (Fig. 3.2). The slope of this linear response is a measure of the efficiency of use of dietary amino acids for protein synthesis. Endocrine manipulations to affect growth could act by increasing the maximal response plateau of growth, which would increase the requirements for dietary amino acids. Alternatively, the efficiency of amino acid use for protein synthesis could be increased, which would not dramatically increase

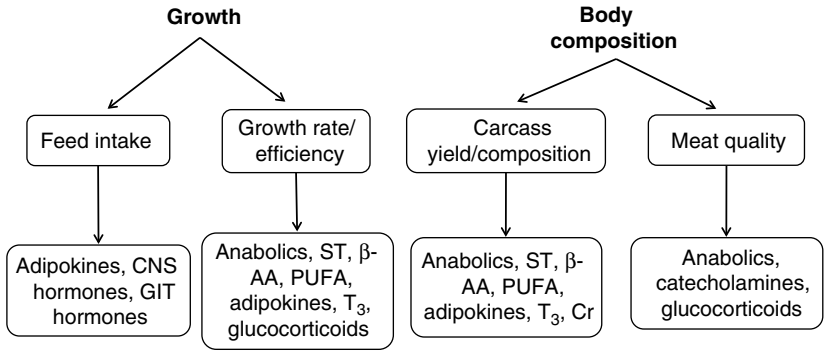


Fig. 3.1. Summary of hormones affecting growth and carcass composition. ST, somatotrophin; β-AA, β-adrenergic agonists; PUFA, polyunsaturated fatty acids; T_3, triiodothyronine; Cr, chromium.

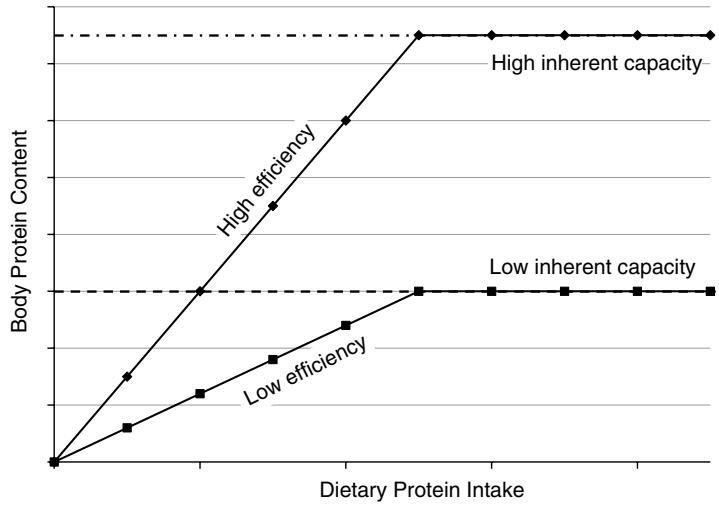


Fig. 3.2. Effects on efficiency versus inherent capacity for protein deposition.

the dietary requirements of amino acids. Increased efficiency of utilization of dietary amino acids may occur through a decrease in amino acid catabolism in the liver, which can be measured as a decrease in levels of plasma urea nitrogen.

The effects of endocrine manipulations on increasing protein synthesis versus decreasing protein catabolism have been determined *in vivo* using labelled amino acids. The dilution of isotope and uptake of amino acids can be measured by arteriovenous differences (sampling blood from the artery that supplies a tissue and the venous blood that leaves the tissue), along with blood flow measurements across the hind limb of an animal. The release of free amino acids produced from protein catabolism would result in a dilution of the specific activity of the labelled amino acid in the venous blood. Uptake of amino acids for protein synthesis would be measured as a decrease in the amount of labelled amino acids in the venous compared with the arterial blood, with no change in specific activity.

Genetics plays an important role in the hormonal response of an individual animal and a large proportion of the genetic selection effort in meat animals has been directed to improving muscle development and the partitioning of nutrients away from fat. This has been more successful in poultry and pigs, partly because of the shorter generation time, compared with cattle. However, selection for increased muscle mass has resulted in some metabolic problems. For example, selection for increased breast muscle in turkeys and broilers has led to problems with pale, soft, exudative (PSE) meat (see Section 3.12) and various cardiovascular problems, such as round heart disease and sudden death syndrome. Selection for decreased fat in pigs has reduced some organoleptic qualities and affected flavour and eating quality. Selection for increased muscle development in certain breeds of pigs caused an increase in porcine stress syndrome (PSS). Selection for double muscling in cattle results in pale meat due to lower myoglobin and fat contents, and double-muscled cattle are more susceptible to stress. It is important to include meat quality and health status attributes in selection programmes to reduce these problems.

Muscle development

An understanding of the regulation of muscle growth (myogenesis) is necessary in order to be able to improve muscle mass without compromising meat quality. Skeletal muscle comprises myofibres that have various contractile and metabolic properties and these are imbedded in connective tissue. The number of fibres determines the growth potential of the animal and this is fixed either late in gestation or in early postnatal life. There are three stages of myogenesis: embryonic and fetal development, when myoblasts proliferate and differentiate into myotubes and myofibres; perinatal growth; and postnatal growth to maturity. Since myogenesis, adipogenesis and fibrinogenesis mainly occur during fetal development, maternal nutrition can affect the composition and function of skeletal muscle and adipose tissue in the offspring.

Myofibres develop in two or three waves during development and the number of myofibres may be regulated by factors affecting both the rate of proliferation and the rate of differentiation of the myoblasts. The first wave comes from the embryonic myoblasts and gives rise to the primary muscle fibres; the second wave comes from the fetal myoblasts and uses the primary fibres as a scaffold to produce the secondary myofibres. During postnatal development, the cross-sectional area of the fibres increases from hypertrophy as satellite cells proliferate and fuse with the myofibres. The muscle fibres and the nuclei within them have lost the ability to divide, so these satellite cells provide the nuclei necessary for growth of the fibres. In older animals, the satellite cells decrease in number and become quiescent, which leads to a plateau of muscle growth. Satellite cells are activated to proliferate, differentiate and fuse with pre-existing muscle fibres to repair damage in injured muscle.

Individual muscles can contain a mixture of different myofibres, which affects their function and metabolism but also affects the eating quality and tenderness of the meat. In mixed or slow-twitch muscles, primary myofibres mature to slow type I oxidative myofibres, while secondary myofibres mature to either fast type II glycolytic or type I fibres. In fast-twitch muscles, only type II fibres are produced. Genetic selection for rapid growth and leanness increases the proportion of glycolytic myofibres. The relative proportions of myofibres are also affected by physiological, nutritional and hormonal factors.

Studies on the hormonal regulation of muscle development have used a number of immortalized muscle cell lines derived from rat or mouse. In addition, primary muscle cultures isolated from

animals at different stages of development can be grown to near confluence and then induced to differentiate into multinucleated myotubes. These cell culture systems can be used to measure the rate of cell proliferation, DNA synthesis and differentiation to express muscle-specific proteins. The rate of protein synthesis and degradation can also be measured using radiolabelled amino acids. See also the discussion of cell culture systems in Section 2.1

For interest

Myostatin, a growth and development factor and member of the TGF-β superfamily, is expressed in developing and adult muscle and suppresses satellite cell activation and self-renewal. Double muscling in cattle is due to a naturally occurring mutation of myostatin which results in greatly increased muscle mass.

Adipose tissue development

Adipose tissue has a considerable capacity to expand, compared with other tissues, and it plays a key role in the regulation of energy balance. The primary function of white adipose tissue is to store excess energy as triacylglycerol and release it when energy is limiting. It also secretes a number of hormones (adipokines) that regulate energy metabolism, reproduction and immune function (see Section 3.5). In contrast, brown adipose tissue is associated with thermogenesis, as a result of the expression of distinct genes such as uncoupling protein-1 (see Section 3.6). The development of brown adipose tissue takes place mainly during embryonic and fetal development, while white adipose tissue development is a continuous process throughout life. The adipomyokine irisin has been suggested to convert white adipose tissue to brown adipose tissue (see Section 3.5).

Adipogenesis is the process in which preadipocyte stem cells develop into functioning adipocytes. This has been studied in several adipose-like cell lines and in stromal–vascular cells isolated from adipose tissue. These cells divide until they are confluent, differentiate to acquire the enzymes and transporters required for accumulation and mobilization of triacylglycerols, and then fill with lipids. Growth of adipose tissue results from growth and proliferation of adipocyte precursor cells as well as increase in size of the mature fat cells. In addition to adipocytes, adipose tissue also contains a number of other cell types, including endothelial cells, pericytes, monocytes/macrophages and nerve cells.

Adipose tissue is found in different depots, including in the abdominal cavity (omental or visceral fat), under the skin (subcutaneous fat) and within the muscles (inter- and intra-muscular fat), and these depots differ in their properties. Intramuscular fat (marbling) is a major factor affecting the flavour and juiciness of meat. Visceral and subcutaneous adipose tissues develop first, at the end of the first trimester in ruminants. Intramuscular adipocytes develop during the late fetal stage to about 250 days of age in cattle. Fibrogenesis is also active in late gestation, which generates connective tissue and cross-linking of collagen in skeletal muscle and contributes to meat toughness.

Bone development

Proper bone development is essential to physically support the increased growth of muscle and adipose tissue. Increased growth due to improved genetics and nutrition has led to increased incidences of skeletal abnormalities in domesticated animals. For example, osteochondrosis in pigs is caused by a lack of ossification of the growth plate of load-bearing bones. Treatment of growing pigs with porcine somatotrophin (pST) (see Section 3.4) can result in cartilage damage and decreased bone strength. Abnormal skeletal development such as tibial dyschondroplasia from accumulation of cartilage in the growth plate of the tibia and femur is a major cause of mortality and downgrading of broiler chickens. This problem is more prevalent in meat-type poultry than in laying hens and is probably linked to rapid growth. These problems lead to major economic losses to the livestock industry and cause significant animal welfare issues.

Bone is a highly specialized form of connective tissue consisting of 70% mineral and 30% organic content, with three major types of cells. Osteoblasts produce the organic extracellular matrix (ECM) or bone matrix consisting primarily of type I collagen, along with other proteins and glycoproteins. This matrix becomes mineralized with crystalline deposits of calcium and phosphate to form the majority of the bone mass. When the osteoblasts are surrounded by the mineralized matrix, they differentiate into mature osteocytes, which are responsible

for the maintenance of the bone. Osteoclasts are large multinucleated phagocytes that are responsible for the resorption of mineralized bone that is required for growth, remodelling and repair of fractures. To achieve this, osteoclasts produce H^+ via carbonic anhydrase to solubilize the mineral components, and proteases to degrade the protein components of the matrix.

The different types of bone cells communicate with each other to maintain a balance between bone formation and resorption (Fig. 3.3). The cytokine receptor activator of NF-κB ligand (RANKL), a member of the tumour necrosis factor family, is located on the membrane of the osteoblast precursor cell and is released by proteolytic cleavage. It binds to the RANK receptor found on the osteoclast precursor cell to stimulate osteoclast maturation and activation leading to bone resorption.

Bone resorption is modulated by osteoprotegerin (OPG), a cytokine produced by bone marrow cells called megakaryocytes. OPG is a RANKL homologue that competes for binding to the RANK receptor to decrease the activation of osteoclasts, thus preventing excessive bone resorption. OPG expression is stimulated and RANKL expression is decreased by oestrogen, which stimulates bone production and prevents resorption, and is a possible treatment for osteoporosis.

Aside from providing structural support, bones provide a reservoir for several minerals, including calcium, phosphate, magnesium, sodium and carbonate. These minerals are in equilibrium with blood mineral concentrations and are mobilized from or deposited in the bone matrix, depending on the requirements of the animal. For example, calcium required for eggshell formation in chickens and for milk production in dairy cows is mobilized from bone stores by osteoclasts. Parathyroid hormone (PTH) binds to cell-surface receptors on osteoblasts to stimulate production of RANKL and in the kidney to produce 1,25-dihydroxy vitamin D to increase bone resorption and increase calcium levels in blood (see Sections 4.1 and 4.2). Another essential function is the production of blood cells by the bone marrow, which also produces a variety of stem cell precursors.

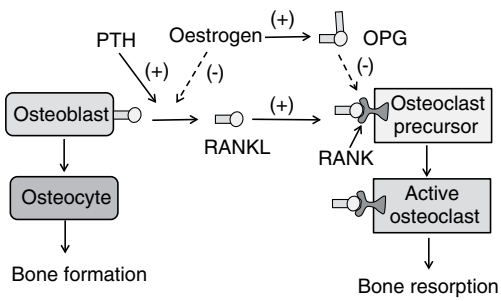


Fig. 3.3. The RANK/RANKL/OPG system for bone remodelling.

3.2 Anabolic Steroids and Analogues

Key concepts

- Anabolic steroids are used to improve nitrogen retention and decrease body fat in a variety of applications.
- Specific structural features of steroids can increase either androgenic or anabolic activity.
- Tissue-specific effects of androgens can be due to further metabolism of the androgens by the 5α-reductase enzyme.
- Anabolic steroids can act by a direct mechanism through the androgen receptor or by indirect methods.
- Indirect mechanisms include being aromatized to oestrogens and acting through the oestrogen receptor or modulating the production of other hormones.
- Anabolic agents can be given to cattle as feed additives, as injections or as implants.
- Safety guidelines have been established for the use of anabolic steroids.

Applications of anabolic steroids

Male cattle, pigs and sheep are traditionally castrated, even though intact (uncastrated) males are more efficient at producing lean meat (muscle) than castrates. Cattle are castrated to reduce the undesirable aggressive behaviour of males and to produce a more consistent carcass with improved tenderness and finish. During sexual development at 8–12 months of age, the collagen content increases in the muscles of bulls, which can decrease meat tenderness. Male pigs are castrated to prevent off-odours and off-flavours (boar taint) in the meat (see Section 3.3); male sheep also tend to have undesirable odours when raised to heavier weights. Female animals tend to produce carcasses with less muscle mass and increased fat content than carcasses from intact males. For these reasons, animals have been

treated with a variety of steroid hormones to improve lean growth or produce more consistent, high-quality carcasses. Females can be treated with androgens to improve muscle growth and decrease carcass fat. Intact males can be treated with oestrogenic compounds to increase carcass fat and meat tenderness and to decrease aggressive behaviour. Castrates can be treated with a combination of oestrogens and androgens to produce the most desirable growth rate and carcass composition.

Anabolic steroids have been used in both human and veterinary medicine for many years. They have been used in the treatment of debilitating conditions such as starvation, and recovery after surgery and from extreme trauma, such as burns. They are used therapeutically in horses to increase appetite and protein retention during recovery from injuries and to reduce the catabolic effects of corticosteroid therapy. They also improve electrolyte retention via decreased urinary losses and increase production of erythropoietin by the kidney, resulting in increased production of erythrocytes. As such, they have been recommended for the treatment of certain types of anaemia.

Anabolic steroids have also been used in competing athletes, such as bodybuilders. The use of anabolic steroids in equine athletes began in the 1960s, but by the 1970s it was decided that the presence of anabolic steroids at the time of racing constituted doping. Anabolic steroids are sometimes illegally used in horses to increase stamina and to modify behaviour, to increase aggression and 'sharpness' for racing. There are few scientific studies and much anecdotal information on the effects of anabolic steroid use in horses. Racing results are affected by a variety of factors, such as track conditions, weather, other horses and the jockey/driver, so determining the effects of doping with anabolic steroids is difficult. Doping in athletes and horses can be detected by analysis of urine and blood, with hair samples used to detect long-term use of anabolics. The World Anti-Doping Agency (WADA) maintains a list of prohibited substances. For more information, see Waller and McLeod (2017) and Fragkaki *et al.* (2009).

A number of different types of compounds, including insulin, growth hormone, β-agonists and steroids, exert anabolic effects to increase nitrogen retention. Steroid hormones currently used as anabolic agents (Fig. 3.4) include testosterone, oestradiol and progesterone, as well as steroid analogues, such as zeranol, and synthetic steroids, such as

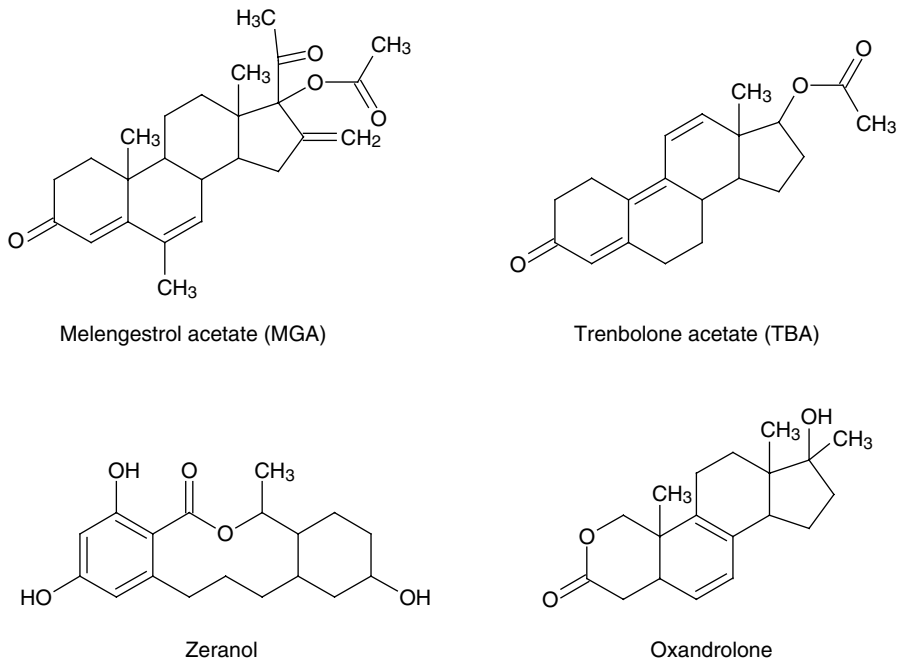


Fig. 3.4. Steroids and steroid analogues used as anabolic agents.

trenbolone acetate (TBA) and melengesterol acetate (MGA). Zeranol is an oestrogenic compound produced from the mycotoxin zearalenone. TBA is a synthetic androgen, which has 10–50 times the anabolic properties of testosterone, with reduced secondary androgenic effects. MGA is an orally active progestagen used for suppressing oestrus and improving rate of gain and feed efficiency in heifers. Corticosteroids can also be administered to finishing animals. Oxandrolone is a synthetic analogue of testosterone that has been used in human studies. For further information about the use of anabolic steroids in animal production, see Reinhardt and Thomson (2016).

Modifications of the structure of testosterone by alkylation at the 17α position produce orally active preparations. However, the most commonly used anabolic steroids in horses have modifications at the C19 or C18 position. These include: nandrolone (decadurabolin or 19-nortestosterone), which is also popular for human bodybuilding; trenbolone, which is used in cattle; and methandienone (methandrostenolone or Dianabol®). Stanozolol (Winstrol®) and boldenone are intended primarily for horses (Fig. 3.5). A number of 'designer' steroids that have modifications to the steroid core have been developed to avoid detection; the first of these were norbolethone, tetrahydrogestrinone and madol.

These compounds can be esterified at the 17α-hydroxyl group and administered intramuscularly in an oil formation for sustained activity. Dosage levels are 0.55–1.1 mg kg^{-1} body weight per injection period. The length of the ester chain affects the rate of absorption and duration of activity. Esters of acetic and propionic acid last for several days; esters of phenylpropionic, cyclopentylpropionic and undecylenic acid last 2–4 weeks; and esters of lauric, decanoic and heptanoic acid last for several months. Once absorbed, the esters are hydrolysed to produce the free steroids. Stanozolol is not esterified, as the pyrazole group on the A ring gives it a 1-week duration of activity.

For use in meat-producing animals, the goal is to maximize the anabolic effects of growth-promoting compounds, while reducing their androgenic effects. The term 'anabolic' refers to the tissue-

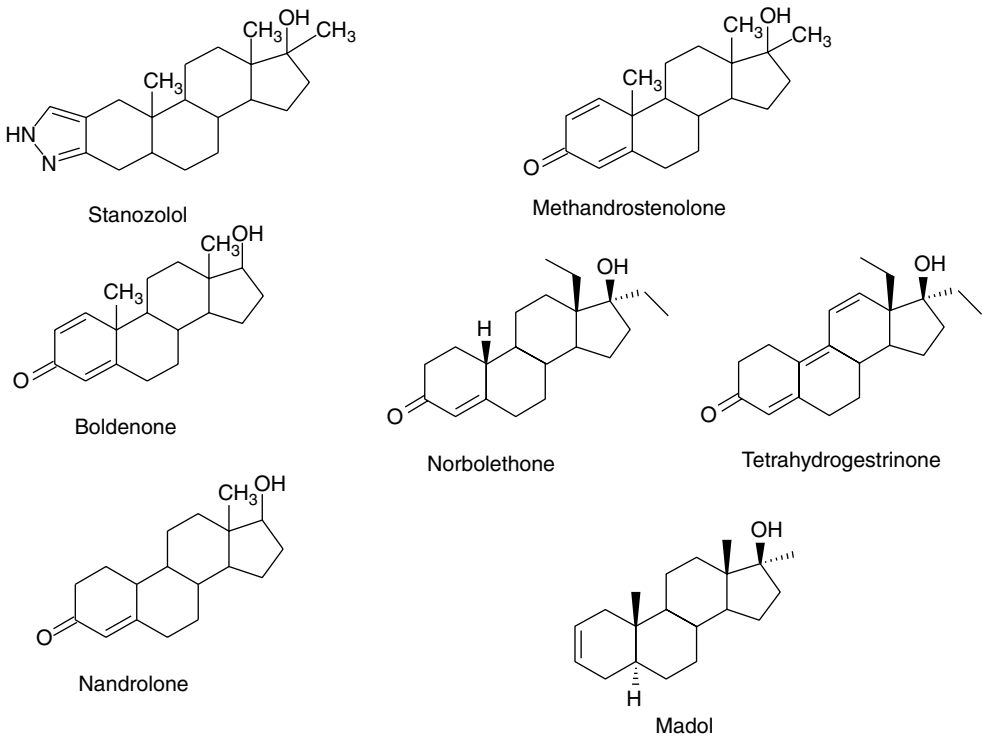


Fig. 3.5. Anabolic steroids used in horses.

building properties of the compound, including the increase in muscle mass (nitrogen retention) and decrease in body fat. The term 'androgenic' refers to the increased development of male sexual characteristics by the compound, including behavioural changes and the development of the reproductive tract. Steroid derivatives have been synthesized with specific androgenic and anabolic activities, which are determined by a bioassay using castrated rats (see Section 2.2). The effects of the compound on the myotropic activity in levator ani muscle is used as a measure of anabolic activity, while the effects on the development of the prostate or seminal vesicles are used as a measure of androgenic activity. The ratio of anabolic to androgenic activity is the Q value or anabolic index for the compound; a value greater than one indicates that the compound is primarily anabolic in nature. These Q values can vary among different laboratories, due to non-standardized methods that are used in their determination. The levator ani muscle may also be an androgen-dependent muscle, so true measures of anabolic activity should be based on whole-body nitrogen retention studies.

The structural features of steroids that are related to anabolic versus androgenic activity are shown in Fig. 3.6. Structure–activity studies have shown that the presence of the 3-keto group, the 5α-hydrogen and the 17β-hydroxyl increases the binding affinity of the steroid to the androgen receptor and increases the androgenic effects of steroids. Removal of the C19 (to form 19-nor steroids) favours anabolic activity and reduces androgenic activity. Oxidation of the 17β-hydroxyl group reduces androgenic activity, and esterification of this hydroxyl group favours anabolic activity. Modification of the A ring by the junction with a pyrazole ring or oxygen atom at C2 leads to a considerable increase in anabolic activity. Anabolic activity can also be increased by alkylation at the 17α- or 7α-position.

Due to the poor oral bioavailability, potential hepatotoxicity and, in particular, the lack of tissue selectivity of some steroids, a large number of non-steroidal androgenic compounds, known as selective androgen receptor modulators (SARMs), have been developed. The earliest of these compounds to be developed were androgen antagonists, but agonists have also been produced. Ongoing research will lead to the development of SARMs with selective preferences for individual tissues or activities. For more information on SARMs, see Solomon *et al*. (2019).

Mechanism of action

Androgens increase the retention of body protein, which can be the result of increased protein synthesis and/or decreased protein degradation. While testosterone increases protein synthesis, trenbolone promotes muscle growth by decreasing protein degradation. Since the number of muscle fibres that an animal has is fixed at birth or shortly thereafter, subsequent muscle fibre growth occurs by hypertrophy rather than hyperplasia. This requires an increase in the number of myonuclei, which come

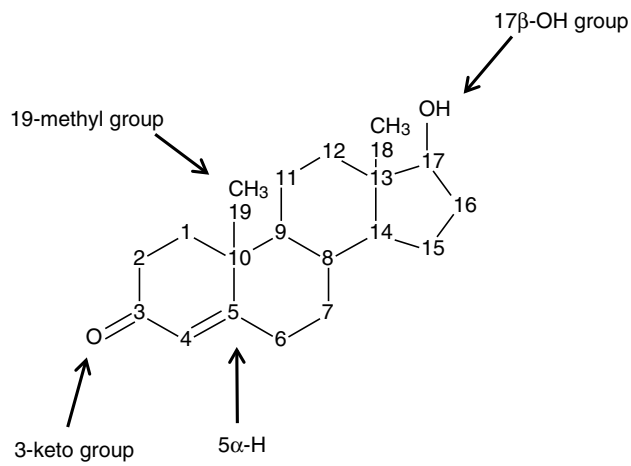


Fig. 3.6. Structural features of steroids affecting androgen and anabolic activities (adapted from Fragkaki *et al*., 2009).

from satellite cells that fuse with the muscle fibre. Androgen treatment causes a dose-dependent increase in the number of satellite cells.

Anabolic steroids can act by a direct mechanism through interaction with the androgen receptor (AR) or by indirect methods. For direct classical effects, steroids bind to the AR in the cytoplasm, and the hormone–receptor complex enters the nucleus to stimulate the transcription of hormone-specific genes. This increases the synthesis of myofibril and sarcoplasmic proteins to increase the overall muscle mass. There are also non-classical effects that are mediated through a membrane-bound AR and intracellular signalling pathways. Indirect mechanisms include being aromatized to oestrogens and acting through the oestrogen receptor or modulating the production of other hormones, such as growth hormone, thyroid hormone and insulin. For more information, see Rossettia *et al.* (2017).

Direct effects

The classical direct genomic effects of androgens are mediated by interaction with the androgen receptor (AR or NR3C4) (Fig. 3.7). Despite the differences in androgenic versus anabolic activity of various compounds, only one androgen receptor has been identified and cloned. The AR gene is located on the X chromosome and codes for a protein of 918 amino acids. As a member of the nuclear receptor family of transcription factors, it contains protein domains that can activate or repress activity (see Section 1.3). These domains are exposed upon hormone binding to allow interactions with various coactivators or co-repressors. These include CREBP (p300), glucocorticoid receptor interacting protein 1 (GRIP1), androgen receptor co-activator (ARA) 54, 55 and 70 and transactivator protein (TAT) interacting protein (Tip60). When AR binds its ligand, it dimerizes, translocates to the nucleus and binds as a homodimer to androgen response elements (AREs) to activate or inhibit expression of many genes. The binding of these co-activator proteins is believed to enhance the stability of the pre-initiation complex. Some factors (CREBP/p300) have intrinsic histone acetyl transferase activity, which will disrupt nucleosome structure and increase the rate of transcription initiation (see Section 1.3).

In addition to the classical genomic effects of the androgen receptor, rapid non-classical effects have also been reported (see Section 1.3). These rapid effects involve the interaction between a membrane-bound AR (mAR) and cytosolic proteins from different signalling pathways and are mediated by changes in intracellular calcium. The mAR involved in these non-classical effects is suggested to be a G protein-coupled receptor acting through the G protein Gnα11. Activation of this membrane-bound receptor leads to activation of the MAP-kinase pathway and the CREB transcription factor to activate gene transcription. This non-classical testosterone signalling is important in the development of Sertoli cells, prostate cells and oocytes, the suppression of the immune response, vasodilation by relaxation of smooth muscle and hypertrophy of skeletal muscle. For more information, see Shihan *et al.* (2014).

The differential effects of androgens in various tissues may be due, in part, to differences in the

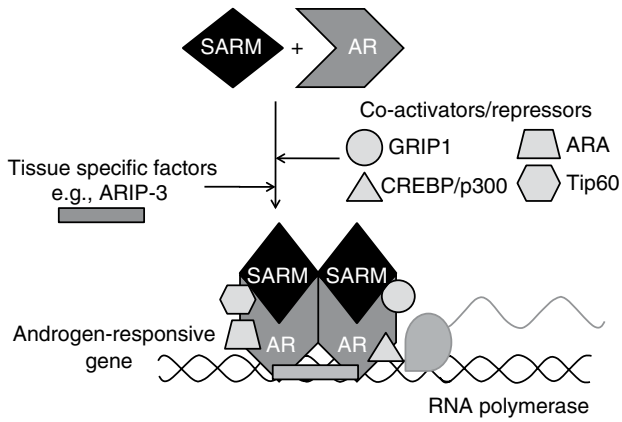


Fig. 3.7. Classical mechanism of action of the androgen receptor.

levels of co-factors that affect the AR, or the presence of unique AR interacting proteins such as ARIP-3, which is specific to testis. In addition, the binding of a particular ligand to the AR may cause a unique conformational change that exposes particular domains that allow the interaction of specific co-factors. This would lead to the selective regulation of individual genes in specific tissues by the AR.

Tissue-specific effects of androgens can also be due to further metabolism of the androgens within different tissues. The 5α-reductase enzyme (SRD5A) exists as three isozymes: SRD5A1 and SRD5A2 are mainly involved in the metabolism of steroid hormones and SRD5A3 is involved in protein glycosylation. Both SRD5A1 and SRD5A2 are located in the endoplasmic reticulum membrane and utilize nicotinamide adenine dinucleotide phosphate (NADPH) for the Δ4 reduction reactions. SRD5A1 prefers 4-androstene-3,17-dione as substrate to generate 5α-androstanedione. SRD5A2 converts testosterone to the more potent androgen, 5α-dihydrotestosterone (5αDHT), which effectively amplifies the androgenic effects. SRD5A2 is expressed at high levels in prostate but not in muscle and bone, so that the predominant androgen in prostate is 5αDHT, while testosterone is the major circulating androgen and the major hormone for androgen action in muscle. Increased numbers of ARs are found during muscle hypertrophy seen with increased exercise or treatment with oxandrolone (see Fig. 3.4), which is a synthetic analogue of testosterone that cannot be aromatized to oestrogens. AR numbers are highest in sex-dependent muscles such as the male rat levator ani muscle, followed by fast-twitch muscles with high glycolytic (anaerobic) capacity, and are lowest in slow-twitch muscles with highest oxidative (aerobic) capacity.

Testicular feminization (Tfm), or androgen insensitivity syndrome (AIS), is an X-linked recessive gene defect in the AR that results in a male pseudohermaphrodite. Virtually all androgen-responsive tissues in Tfm individuals are not responsive to testosterone, due to the lack of a functional AR. Female carriers have the Tfm gene defect on one X chromosome and a normal AR on the other chromosome. These carriers have an androgen responsiveness that is intermediate between normal and Tfm animals.

Indirect effects

Indirect mechanisms of androgen action include the androgens being aromatized to oestrogens and acting through the oestrogen receptor or by modulating the production of other hormones, such as growth hormone, thyroid hormone and insulin.

The aromatization of androgens to oestrogens by the aromatase enzyme (CYP19A1) is important in some tissues, such as in adipose tissue, bone and the central nervous system (CNS). The aromatase reaction can be blocked by removal of the C19 methyl function from the androgen (as in nandrolone) or modification of the A ring (as in oxandrolone and stanozolol). There are two forms of the oestrogen receptor, ERα and ERβ, which respond to different ligands and have different tissue distribution patterns and physiological roles. ERα is the most important isoform in skeletal muscle of cattle. There is also evidence of a G protein-coupled membrane-bound oestrogen receptor (GPER1) in the endoplasmic reticulum of skeletal muscle. A number of selective oestrogen receptor modulators (SERMs) have been developed that interact with these different receptor isoforms. For more information see Komm and Mirkin (2014) and Fuentes and Silveyra (2019).

Androgens can also function indirectly by increasing the production of somatotrophin (ST) or insulin-like growth factor-1 (IGF-1), and prior exposure to androgens can prime cells for the secondary actions of IGF-1 (see Section 3.4). Glucocorticoids may increase the production of IGF-binding proteins to decrease IGF bioactivity. Anabolic steroids also reduce the production of luteinizing hormone (LH) and testosterone, delay sexual maturation and reduce testicular growth and spermatogenesis in bulls.

There appears to be a reciprocal relationship between testosterone (an anabolic hormone) and cortisol (a catabolic hormone) in bulls. Anabolic steroids could potentially act as antagonists of the catabolic action of glucocorticoids, by decreasing the concentration of the glucocorticoids or by displacing the glucocorticoids from their receptors. Studies *in vitro* have shown that testosterone has a high affinity for the glucocorticoid receptor and could thus prevent the normal protein catabolic action of glucocorticoids.

There is some evidence that anabolic agents with oestrogenic activity increase the blood levels of insulin to promote growth. Oestrogens increase growth rate and feed efficiency in ruminants without the loss of desired carcass characteristics. Insulin has anabolic effects on adipose and skeletal muscle, while thyroid hormone (T_3) has a

catabolic effect on adipose tissue and an anabolic influence over skeletal muscle.

Delivery systems

Anabolic agents can be given to cattle in three ways:

1. Oral administration by addition to the feed. This requires an orally active hormone (e.g. MGA) and higher levels are needed to stimulate growth performance.
2. Repeated intramuscular injections can be used, but this is labour intensive and results in high levels of hormones at the injection site.
3. Implants containing the steroids can be used for sustained release of the hormone. Implants are normally placed between the skin and cartilage in the middle one-third of the back-side of the ear, which is a desirable site since it is eliminated after slaughter. Steroids can be formulated into a compressed pellet that has a lifespan of 90–120 days of activity before completely degrading. Silastic rubber implants can also be used to provide a slow and continuous release for 200–400 days. Some implants also contain an antimicrobial such as oxytetracycline or tylosine tartrate to provide a local antibacterial effect. For a discussion of the different methods of hormone delivery, see Section 2.4. Some commercially available implants are listed in Table 3.1.

The general principle for use of anabolic steroids is to supplement with the hormone that is deficient for the particular animal. Implants can enhance growth rate in suckling calves by 4–8%, in growing calves by 10–20% and in finishing cattle by 15%. The expected improvement in feed efficiency is 6–8% in growing cattle and 8–10% in finishing cattle. Growth responses are likely to be greater in steers than in intact males or females, which already have a source of endogenous hormones. Androgenic compounds (trenbolone acetate) are more effective in females, while oestrogens (oestradiol and zeranol) and progesterone are more effective in males. A combination of androgen and oestrogen or progestagen and oestrogen is given to castrated males.

Table 3.1. Steroid anabolic agents used in some commercially available implants.

Trade name	Chemical name
Compudose®	Oestradiol 17β
Ralgro®	Zeranol
Synovex-S®	Oestradiol 17β benzoate + progesterone
Synovex-H®	Oestradiol 17β benzoate + testosterone propionate
Synovex-Plus®	Oestradiol 17β benzoate + trenbolone acetate
Finaplix®	Trenbolone acetate
Revalor®	Oestradiol 17β + trenbolone acetate

A combination of androgen and oestrogen can be given to intact males to inhibit testicular function. Implanting zeranol in bulls from birth to slaughter decreases carcass masculinity, improves fat cover to an acceptable finish and improves meat quality and palatability. Steers implanted with trenbolone and oestradiol have weight gains similar to intact bulls. Treatment with a combination of androgen and oestrogen or androgen alone increases protein accretion and decreases fat content. Treatment with oestrogens or an oestrogen/progestin combination increases fat deposition, particularly in uncastrated males. Treatment of bulls with TBA and oestradiol increases tenderness and lowers the connective tissue content, while not affecting the juiciness and flavour of steaks.

A conservative approach using mild oestrogens ensures marbling with a slight growth response. An intermediate approach using a mild combination of androgens and oestrogens gives improved growth with a slight depression of marbling. An aggressive approach using a strong combination of androgens and oestrogens gives maximum improvement in growth with very poor marbling, negative effects on finishing and an increased tendency for 'dark cutting' or dark, firm and dry (DFD) beef, due to lower glycogen levels (see Section 3.12). Mild oestrogens include zeranol, which produces a 5% decrease in marbling, and the combination of oestrogen and progesterone, which gives a 10% decrease in marbling. Treatment with the strong androgen TBA gives a 20–25% decrease in marbling.

The efficacy of growth promoters is also affected by nutrition. Cattle implanted with anabolic steroids require higher levels of dietary protein or nitrogen to sustain the higher growth rates. The level of response may be dependent on the initial nutritional status, level of live weight gain, age, dose and length of treatment. Higher response is found in cattle that are already growing at a rapid rate, since growth in each animal is limited by its genetic potential. The average daily gain (ADG) should be higher than 0.6 kg day^{-1} for calves and 1.8–2.0 kg day^{-1} for growing cattle for anabolic treatment to be effective. Anabolic treatment does not affect the digestibility

of feed, although feed intake and feed efficiency may increase. Sufficient levels of dietary protein and essential amino acids are needed to sustain the higher growth rates. Anabolic agents are ineffective in early treatment of intact males, while castrates and females can be treated with anabolic agents at any age. The response to repeat implants is reduced with each sequential implant. Intramuscular injection of synthetic hormones must be repeated every 10–15 days for 8–10 weeks.

Weights of the liver and spleen are increased in steers implanted with anabolic agents. Treatment with corticoids alters thymus, thyroid and adrenal glands and lowers the levels of lymphocytes and neutrophils. These alterations in the immune system can result in health disorders, such as respiratory illness or infection in finishing animals. Treatment of intact males with oestrogenic compounds decreases testicular maturation and spermatogenesis. Growth-promoting implants are not recommended for replacement heifers because of possible detrimental effects on fertility. For more information, see Smith and Johnson (2020).

Anabolic agents produce similar responses in growth and carcass composition of sheep as in cattle. The results in pigs have been mixed, with few consistent beneficial effects found. Anabolic treatment of fish with methyltestosterone is used to induce sex reversal and promote growth (see Section 5.2). Salmon treated with methyltestosterone had 20–33% higher daily gain, 18–19% improved feed conversion ratio and 19–34% lower ammonia excretion than untreated fish.

Safety issues

Anabolic agents have had a very significant impact on beef production in North America since they were first licensed for use in the early 1950s, and today at least 90% of all fed cattle in the USA are implanted. In the early years, the synthetic stilbenes diethylstilboestrol, hexoestrol and dienoestrol were used. These were subsequently banned in the EU, the USA and Canada because of their potential carcinogenic activity. Since December 1988, there has been a complete ban on the use of hormonal-type growth promoters in the EU. Consumers generally consider the use of hormones in meat production to be a very high risk. However, in 1999 the Food and Agriculture/World Health Organization (FAO/WHO) Joint Expert Committee on Food Additives (JECFA) reviewed the use of oestradiol, progesterone and testosterone in meat production and declared that they were safe. The 'no observed effects limits' (NOELs) for oestradiol, progesterone and testosterone are 5 μg kg^{-1} body weight (BW), 3.3 mg kg^{-1} BW and 1.7 mg kg^{-1} BW, respectively. Using a safety factor of 100, this limits the acceptable daily intake (ADI) to 50 ng kg^{-1} BW, 33 μg kg^{-1} BW and 17 μg kg^{-1} BW for oestradiol, progesterone and testosterone, respectively. The theoretical maximum daily intakes for these compounds can be calculated from data on tissue levels of these compounds in treated animals and considering that an individual would consume 300 g muscle, 100 g liver, 50 g kidney and 50 g fat per day. These calculations show that the theoretical daily maximum intakes are substantially less than the ADI from consuming meat from treated animals. For more information, see Jeong *et al*. (2010).

The ADI values for oestradiol used by the FAO/WHO have been questioned based on the new data for oestrogen production and clearance in prepubertal boys, with an ADI of 0.04–0.1 μg day^{-1}. This implies that the additional dose of oestrogen from consuming meat from implanted animals could equal the production rate in prepubertal boys. However, levels of oestradiol 17β are significantly higher (about 60×) in meat from animals in late pregnancy than from steers with a hormonal implant. There are also high levels of natural steroids in meat from intact males and in the milk from pregnant cows. Levels of natural oestrogens found in plants and plant products, such as soybean oil, are several orders of magnitude higher than in beef from implanted animals (see Section 6.4).

The maximum residue levels (MRLs) for TBA, zeranol and MGA are 2.0 μg kg^{-1} muscle, 70 μg kg^{-1} and 25 μg kg^{-1} tissue, respectively. A 48 h withdrawal time for MGA is prescribed. The US Department of Agriculture (USDA) and Agriculture and Agrifood Canada (AAFC) maintain drug testing programmes. There were some instances of high levels of zeranol in 1988 and 1989, but otherwise tests for zeranol, TBA and MGA were negative between 1988 and 1993.

Detection of anabolic steroid use in horses is based on levels in urine or blood, with initial screening by immunoassays and confirmation by gas chromatography/mass spectrometry (GC/MS). However, colts naturally produce testosterone and 19-nortestosterone, so it is more difficult to detect use of these steroids. A ratio of oestrane-3,17-diol to 5(10) oestrene-3,17-diol levels greater than one indicates that doping with nandrolone has occurred.

Anabolic steroid treatment will reduce cycling in mares and sperm production and testis size in stallions. In humans, a number of anabolic steroids have been shown to be hepatotoxic. High doses of anabolic steroids can increase the risk of severe coronary heart disease, decrease high-density lipoprotein (HDL) while increasing low-density lipoprotein (LDL) and reduce the immune response.

3.3 Use of Intact (Uncastrated) Male Pigs for Pork Production

Key concepts

- Castration of pigs to prevent boar taint is widely practised but this reduces production efficiency and animal welfare.
- Boar taint is primarily due to high levels of androstenone or skatole in fat.
- Analytical methods for measuring boar taint are available but technology for on-/at-line detection of boar taint is still under development.
- Skatole levels can be controlled by diet and management, while androstenone levels are affected by sexual maturity.
- Immunocastration is an effective method for controlling boar taint.
- Genetic approaches may be used to produce future lines of boar-taint-free pigs.

Advantages and problems of intact male pigs

Male pigs destined for pork production were traditionally castrated to increase the proportion of fat in carcasses, to reduce aggressive and sexual behaviour, and to reduce the risk of boar taint, an unpleasant smell from heated pork products. With the increased demand for lean meat, only the two latter reasons to castrate remain. However, castration decreases the profitability and increases animal welfare concerns of pork production.

The advantages and disadvantages of raising intact (entire) male pigs compared with castrates are summarized in Table 3.2. Overall production costs are substantially lower for intact males than for castrates. The labour costs for castration are eliminated, along with death losses and temporary decreases in performance usually seen following castration. Intact males have up to 14% better feed conversion efficiency than castrates and grow up to 13% faster than castrates. Entire males eat 9% less feed. As a result, the overall output of nitrogen in manure is less with intact males than with castrates. Intact males may also be more resistant to diseases that negatively affect performance.

Table 3.2. Advantages and disadvantages of raising intact male pigs.

Advantages	Disadvantages
Improved lean content	Boar taint
Improved feed efficiency	Aggressive and sexual behaviour
Faster growth	Potential for DFD or PSE meat
Decreased labour costs and death loss	Potential for over-lean carcass
Improved animal welfare	Higher unsaturated fat content
	Lower dressing percentage

Intact male pigs, compared with other sexes, have a slightly lower dressing percentage and a higher bone content but up to 20% higher lean content and 9–21% less back fat. Decreased back fat in intact males would result in a higher grading for carcasses but carcass grade may be underestimated unless special equations are used for carcass grading systems to account for differences in fat and protein deposition in intact males. The low intramuscular fat content, which can affect the flavour and texture of the meat, may be of some concern in intact males if it is lower than the 2–3% level recommended for optimum sensory quality. Intramuscular fat levels can be higher for gilts than for intact males, but breed differences are more pronounced than sex differences. Meat cuts from intact males are more appealing to the consumer because of the decrease in intermuscular fat.

There are important differences between sexes in the fatty tissue composition as well as in the composition of the lipid fraction of the fat. The back fat from intact males contains more water, more protein and less lipid than that from castrates and gilts. The lipid fraction of intact male back fat contains more unsaturated fatty acids than that of castrates, with that of gilts being intermediate. The high level of unsaturation of intact male fat is mainly due to the higher levels of the unsaturated linoleic (18:2) and linolenic (18:3) acids with a lower level of saturated palmitic acid (16:0).

The lower fat content and different fatty acid composition of carcasses from intact males has its

advantages as well as disadvantages. Intact-male meat, with its lower level of fat and a healthier balance of fatty acids, offers a greater nutritional value and may be more appealing to consumers. The leanness of the meat has been found to be the most important visual criterion in purchasing pork. On the other hand, fat from intact males is softer due to the high level of unsaturation and more easily separates from the other tissues. This causes difficulties in handling and processing and decreases the quality of the cuts. Also, since unsaturated fatty acids are more susceptible to oxidation, intact-male fat turns rancid faster than that of gilts and castrates. However, the degree of saturation of fatty acids in the intact-male fat can be decreased by reducing the level of unsaturated fatty acids or increasing the energy level in pig diets. The poorer quality of fat in boars is a result of the leanness of the animals and thus is more important in very lean genotypes.

Intact males are considered to be more aggressive and therefore potentially more susceptible to the development of PSE (pale, soft, exudative) and especially DFD (dark, firm and dry) conditions in meat. Thus, care should be exercised during pre-slaughter handling of intact males. Avoiding mixing of unfamiliar animals, minimizing lairage time and using good handling practices would reduce the amount of stress and consequently the incidence of DFD. Such measures would also reduce the incidence of skin damage, which is another problem associated with higher aggressiveness of intact males. Fighting sometimes results in only superficial skin blemishes but in other cases it can cause major carcass bruising and consequently financial losses from increased carcass trim. The incidence of carcass and skin damage varies considerably between slaughter plants and major problems are likely to occur where good pre-slaughter practices are not exercised. For more information on meat quality in intact male pigs, see the review by Škrlep *et al.* (2020).

Although the problems with meat quality may be significant, particularly in lean strains of pigs, the main reason that prevents raising of intact males is the presence of boar taint. In many countries, including Canada, all male pigs intended for meat production are castrated at a very young age to avoid the potential consumer dissatisfaction from boar taint in the carcasses. Under the current Canadian pork carcass grading system, a carcass from an intact male pig (boar) is not graded and is assigned an index of 67. This compares with a normal grade of 100 for a market hog, making boar carcasses worth about one-third less than a similar carcass from a gilt or castrate.

The 28 EU countries (reduced to 27 when the UK left the EU in 2020) produce 250 million slaughter pigs per year, with the eight largest producers accounting for 81% of all pigs in Europe. These are: Germany (24%), Spain (16%), France, Poland and Denmark (about 10% each) and the Netherlands, Italy and Belgium (about 5% each). Traditionally more than 90% of male pigs in the UK and Ireland are not castrated, with about 60% of male pigs in Spain, Portugal, Greece and the Netherlands, 12% in Germany and France and 5% in Denmark raised uncastrated. The vast majority of castrations are performed by farmers and without anaesthesia or analgesia but in Norway, Switzerland and the Netherlands castration is now performed with anaesthesia. For more information, see Bee *et al.* (2015) and Aluwé *et al.* (2020).

Particularly in European countries, animal welfare concerns are putting more and more pressure on the pig production chain to abandon castration. Avoiding surgical castration without anaesthesia undoubtedly improves the welfare of the animals in the short term, although increased aggression may occur with intact males as they approach market weight. As the males approach sexual maturity, there can also be excessive sexual activity such as mounting, which results in decreased feed consumption and poorer growth performance. Avoiding castration would also lower the costs of pig breeding programmes, since the breeding stock could be selected at an older age and the non-selected animals could be sold for meat at a normal market price.

Effects of sex steroid hormones

The natural sex steroid hormones produced in the testes account for the increase in growth rate and lean meat yield of intact male pigs compared with castrates. The most predominant androgens in the testis of the pig are 5α-androstanediol, dehydroepiandrosterone (DHEA), 19-nortestosterone and testosterone. These steroids (and their metabolites 5α-dihydrotestosterone (DHT) and oestrogen) stimulate the development of the reproductive tract and secondary sex characteristics, including actions in the central nervous system (see Section 5.1). In addition, these steroids have dramatic anabolic

effects to stimulate muscle growth, nitrogen retention, phosphorus retention and bone growth, and cause the redistribution of nutrients away from the synthesis of subcutaneous fat. This results in an enhanced growth rate and feed conversion efficiency along with a reduced back fat thickness and increased lean content of carcasses from intact males compared with females or castrates.

The biological effects of androgens are mediated by their interaction with specific receptors inside the cells of the target tissues. In some tissues, DHT is the active androgen that binds to the receptor, while in other tissues it is testosterone. Synthetic anabolic steroids such as nandrolone decanoate, oxandrolone and stanozolol have high anabolic activity in skin, muscle and bone but have minimal androgenic activity. These effects may be due to competition between these anabolic steroids and endogenous glucocorticoids for their receptors in non-reproductive tissues (see Section 3.2). Anti-androgen compounds, such as cyproterone acetate and flutamide, act by binding to the androgen receptor, thus preventing binding of the active androgen. Oestrogen has also been shown to be involved in the development of secondary sex characteristics in the boar.

The testicular synthesis of both the sex steroid hormones and the 16-androstene steroids (responsible for boar taint) is induced *in vivo* by administration of LH and human chorionic gonadotrophin (hCG) and by sexual stimulation. The production and secretion of testosterone by the testis is controlled by the release of LH from the anterior pituitary gland, which in turn is regulated by gonadotropin-releasing hormone I (GnRH-I) produced by the hypothalamus (Fig. 3.8). As the animal approaches sexual maturity, levels of GnRH rise and stimulate the release of LH into the circulation. The binding of LH to specific receptors on the surface of the Leydig cells in the testis stimulates the synthesis of steroidogenic enzymes and thus increases the production of androgens and 16-androstene steroids. Steroid hormone production can be blocked by the use of GnRH agonists, which cause a down-regulation of the number of GnRH receptors, and with GnRH antagonists, which block the binding of GnRH-I to its receptor. Analogues of LH that block LH action also cause decreased production of both 16-androstene steroids and androgens. Production of antibodies that bind GnRH or LH and thus inhibit their activity, so-called 'immunocastration', will also result in decreased 16-androstene steroid and androgen production.

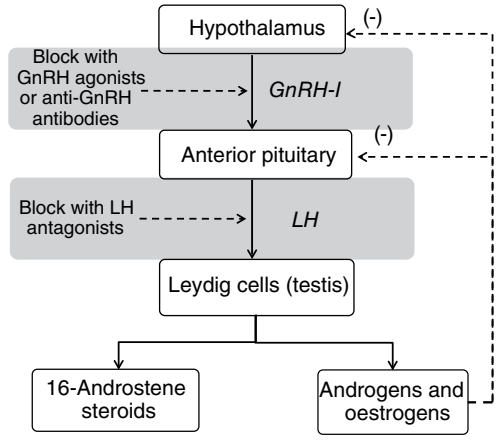


Fig. 3.8. Regulation of testicular steroid biosynthesis.

A second form of GnRH, known as GnRH-II, has been identified in many species, but pig is the only livestock species that has a functional form of GnRH-II and its receptor. GnRH-II is 70% homologous with GnRH-I and these different forms of GnRH and their respective receptors are coded by different genes. GnRH-I is expressed mainly in the brain while GnRH-II is expressed in peripheral tissues. GnRH-II binds to its receptor on Leydig cells to increase production of sex steroids independent of LH, and immunization against GnRH-II reduces testosterone but not gonadotrophin secretion. LH secretion is also not reduced in transgenic pigs that have a non-functional GnRH-II receptor. Thus, GnRH-II functions as an autocrine/paracrine regulator of steroidogenesis in the testis of the boar. For more information, see White *et al.* (2022).

Description of boar taint

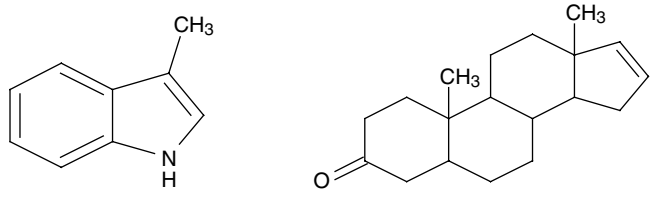
Boar taint is the presence of off-odours and off-flavours found predominantly, but not exclusively, in the meat of some intact male pigs. The main compounds responsible for boar taint (Fig. 3.9) are 5α-androst-16-en-3-one (androstenone) and 3-methylindole (3MI, also known as skatole). There is some evidence that they cannot completely account for the occurrence of boar taint as determined by a trained sensory panel. Intact males near sexual maturity, as judged by the length of the bulbourethral glands, which have low levels of androstenone and skatole, still had significant boar taint odour and flavour scores. It may be that other factors related to sexual maturity also contribute to

boar taint. For example, 4-phenyl-3-buten-2-one and short-chain fatty acids have been suggested to play a role in the overall perception of boar taint.

Androstenone is a 16-androstene steroid that is synthesized in the testes and is released into the bloodstream as both the free steroid and as androstenone sulfate (Fig 3.10). Androstenone is removed by a specific binding protein (pheromaxein) in the salivary gland, along with salivary lipocalin (SAL), a member of the lipocalin family (see Section 6.2) and released into the saliva, where it acts as a pheromone, inducing a mating response in oestrous sows. Due to its hydrophobicity, free androstenone also accumulates in adipose tissue, causing a urine-like off-odour when heated. Androstenone is metabolized in the liver through both Phase I (hydroxylation) and Phase II (conjugation) reactions and excreted in the bile (see Section 1.2). Androstenone sulfate can potentially accumulate in lean tissue and be taken up by adipocytes from the plasma and deconjugated to form the free steroid.

Skatole is produced by bacterial degradation of tryptophan in the hind gut. It is absorbed from the gut, metabolized in the liver, partially excreted with the urine and partially deposited in the fatty tissue (Fig 3.11). It produces a faecal-like odour and a bitter taste.

Cut-off levels that define a limit between untainted and tainted samples have been proposed for androstenone and skatole from sensory assessments by trained panels. Cut-off levels for skatole are considered to be 0.20 or 0.25 ppm, while cut-off levels for androstenone are much more difficult to establish, due to the existence of anosmic people, but range between 0.5 and 1 ppm. The incidence of boar taint is very variable, due to the different breeds, slaughter weights and production systems used in different countries. Because of this and also



Skatole Androstenone

Fig. 3.9. Compounds primarily responsible for boar taint.

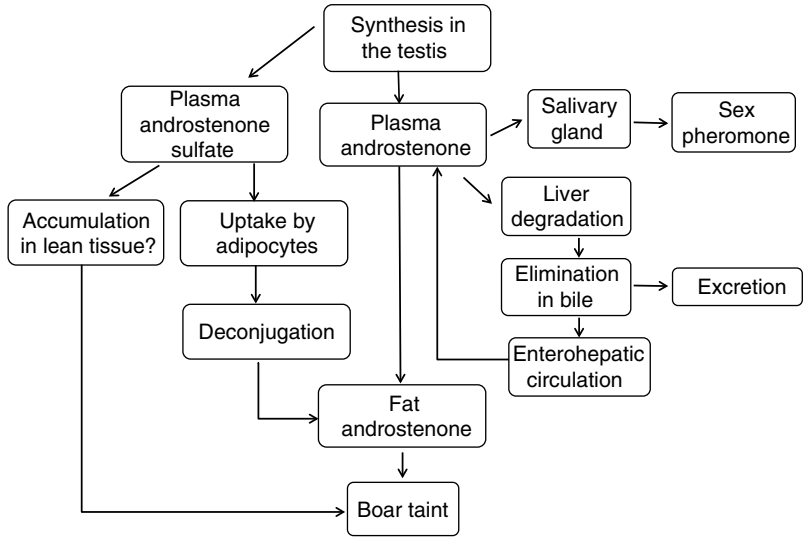


Fig. 3.10. Summary of the metabolic pathways of androstenone.

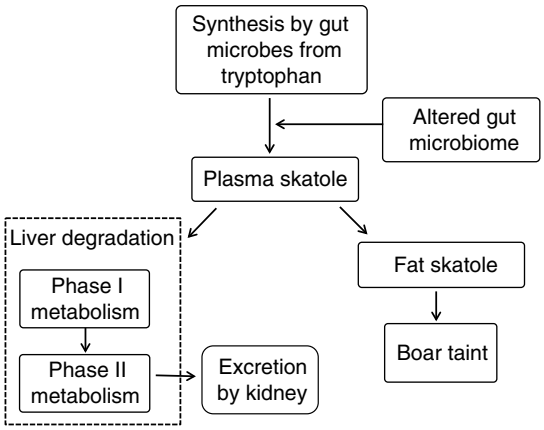


Fig. 3.11. Summary of the metabolic pathways of skatole.

the variety of culinary habits between countries, the acceptability of meat from intact males can be quite inconsistent in consumer surveys. As many as 99% of consumers are sensitive to skatole, whereas around one-third of them are insensitive to androstenone, with more men than women being insensitive and large variations between countries. Some of this variation may be due to differences between individuals in the expression of the human odorant receptor, OR7D4, which is involved in sensitivity to androstenone.

Measurement of boar taint

The availability of quick, cheap and reliable methods for the assessment of boar taint on the slaughter line would enable the sorting of carcasses according to levels of boar taint. A number of different methods have been developed for measuring the levels of skatole and androstenone in carcasses (see Section 2.2 for a discussion of methods for measuring hormones). Immunological methods including radioimmunoassay (RIA) have been developed for androstenone, and ELISAs have been developed for skatole and androstenone. The commercial usefulness of immunological methods can be limited by the long time required to develop equilibrium binding with the antibody and the requirement for extraction of the fat samples. These factors can also introduce a significant amount of variation in these assays, with coefficients of variation of more than 10% commonly found. Rapid lateral flow tests may be a cost-effective alternative that could be used to confirm the effectiveness of taint control methods.

Chromatographic methods, such as high-performance liquid chromatography (HPLC) and GC (see Section 2.2), have been used for measuring skatole and androstenone. Chromatographic methods can have the advantage of measuring a number of related compounds at one time and are usually quite specific and not affected by interfering compounds. However, they can be time consuming, technically difficult, expensive and prone to equipment failure. Careful extraction of the samples is required for chromatographic analysis, both to ensure good resolution of the sample analysis and to maintain the useful life of the chromatography columns. This makes them generally more suitable for use in experimental analysis rather than for the routine analysis of boar taint compounds on a slaughter line.

There are very few methods that are currently available for detection of boar taint on the slaughter line. A colorimetric method for skatole developed in Denmark has been used for sorting carcasses in slaughter plants. However, the equipment has a limited capacity and only measures skatole. It has been replaced by an instrumental method based on mass spectrometric measurement of skatole and androstenone. A sniff test, so-called 'human nose' method, where fat from carcasses is heated on the slaughter line and sniffed by a single expert, are currently in use in the Netherlands, Belgium, Germany and France. This method is cheap but subjective and very dependent on human factors.

The detection of compounds causing taint instantaneously on the slaughter line, using probes such as immunosensors, electronic sensors or chemical sensors, would be ideal. Immunosensors couple antibody–antigen reactions to an electronic signal generated by a transducer. Electronic sensors have been used in 'electronic noses' and are composed of semiconductors whose characteristics change when a particular substance is adsorbed onto the surface. Various types of electronic sensors have been investigated for detection of boar taint and 'artificial noses' have been shown to simulate the response of a laboratory panel to boar taint. Mass spectrometry, fast gas chromatography and gas phase spectrometry have also been investigated to measure boar taint compounds. Major obstacles include obtaining an appropriate sample on the slaughter line and the presence of a large number of volatile

compounds in fat that mask the detection of the boar taint compounds. As such, work is continuing to develop low-cost commercially useful methods for detecting boar taint. For more information on detection of boar taint, see Font-i-Furnols *et al.* (2020).

Use of tainted meat in processed products

Meat with high levels of boar taint is generally categorized as unfit for human consumption by regulations in many countries. However, heat processing can reduce the levels of boar taint in processed meat products, since both androstenone and skatole are volatile. The taint can also be masked by smoking the products or including flavour-masking herbs and spices. Decreasing the amount of tainted fat in the product by diluting with untainted material is also effective. Products produced from tainted meat that were not heated during processing, such as dry sausages, dry-cured hams and cured bellies, were usually evaluated as having a stronger odour than that of controls. However, cooked products, such as cooked hams, luncheon meat, frankfurters and cooked sausages, were acceptable unless they were prepared from very strongly tainted meat. The important factor influencing the acceptability of processed meat from intact males is the way it is consumed. If it is heated prior to consumption, the perception of the odour is enhanced and the taint is more easily detected. For more information, see the review by Škrlep *et al.* (2020).

Metabolism of androstenone and skatole

The extent of skatole and androstenone accumulation in carcasses is controlled by the balance between the processes that produce these compounds and processes that are involved in degrading and removing the compounds. Androstenone is synthesized in the testis and the synthesis increases as the pig nears sexual maturity. Skatole is produced by the bacterial degradation of tryptophan in the gut and is affected by the composition of the gut microbiome and the availability of tryptophan. It is absorbed into the bloodstream and metabolized in the liver.

The 16-androstene steroids (including androstenone) and the androgen sex steroids are synthesized in the testis from pregnenolone in a reaction that is catalysed by cytochrome P450C17A1 (CYP17A1) along with cytochrome b5 (CYB5A) and the associated reductases. The biosynthetic pathway is given in Fig. 3.12. Increased expression of CYB5A increases the amount of 16-androstene relative to androgen synthesis, and mutations that alter the interaction between CYB5A and CYP17A1 reduce the synthesis of 16-androstene steroids. This suggests that decreasing levels or activity of cytochrome b5 in the testis could be used to reduce boar taint from androstenone.

The 5α-reductase enzyme (SRD5A) catalyses the final step in the synthesis of androstenone, which is the reduction of the Δ4 double bond in 4,16-androstadien-3-one. Thus, inhibition of this enzyme could reduce the synthesis of androstenone. However, this enzyme is also responsible for the conversion of testosterone to dihydrotestosterone, which is the ultimate androgen in some tissues. If a specific isoform can be found in pig testis for the synthesis of androstenone, it may be possible to reduce boar taint from androstenone by inhibition of this isoform without affecting the biological actions of androgens. Androstenone is subsequently reduced by 3β-hydroxysteroid dehydrogenase (HSD3B) to produce 3β-androstenol or aldo-keto reductase (AKR1C) to 3α-androstenol.

Androstenone and the androstenols can then undergo Phase II conjugation reactions by sulfotransferase 2A1 to form sulfoconjugates. Approximately 70% of the total androstenone in the circulation is present as androstenone sulfate and this may function as a steroid reservoir, as do many steroid sulfates. Androstenone sulfate is taken up by adipocytes via the organic anion transporting polypeptide B (OATP-B) and is then deconjugated by steroid sulfatase (STS), to form free androstenone (see Fig. 3.10). Androstenone sulfate can potentially also accumulate in hydrophilic lean tissue to contribute to boar taint. This may in part explain the discrepancies between measurements of androstenone concentration in fat, and sensory scores that typically use both fat and muscle tissue. Steroids are also sulfoconjugated in the liver and then excreted into the intestine via the bile. The gut microflora can then remove the sulfoconjugate and the free steroid is reabsorbed; this is called the enterohepatic circulation (see Section 1.2). Feeding binding agents such as activated charcoal can bind the steroids in the gut and prevent their reabsorption. This is a potential method to reduce boar taint from androstenone.

Skatole is produced by gut bacteria which degrade tryptophan that is available from undigested feed or from the turnover of cells lining the

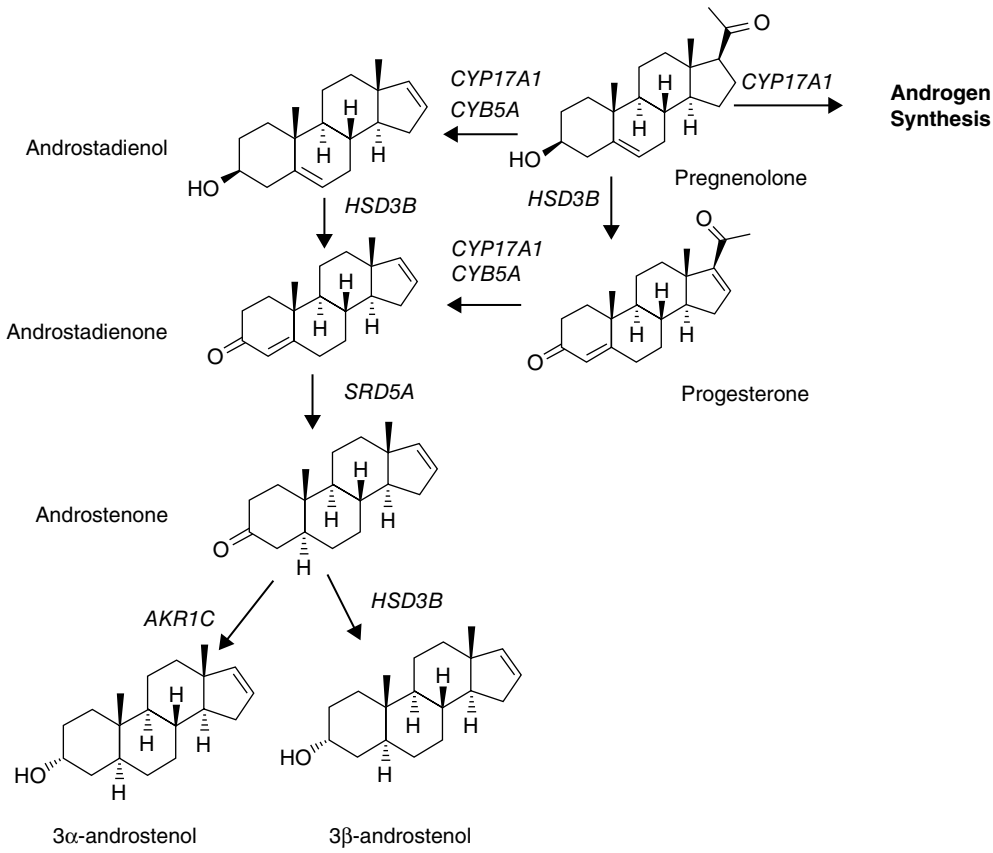


Fig. 3.12. Biosynthesis of 16-androstene steroids.

gut of the pig. The amount of skatole production varies between individual pigs and levels of skatole in the faeces can be used to identify those pigs that produce high levels of skatole. Skatole synthesis is dramatically affected by the composition of the gut microbiome and can occur in four bacterial species of *Clostridium* and *Olsenella* genera (see Section 3.11 for more information on the microbiome).

Skatole is absorbed from the gut and then metabolized primarily in the liver. The major metabolites (Fig. 3.13) that are formed by Phase I metabolism are 6-hydroxyskatole (6-OH-3MI), indole-3-carbinol (I3C), 3-hydroxy-3-methyloxindole (HMOI) and 3-methyloxindole (3MOI). Several cytochrome P450 enzymes are involved in Phase I metabolism of skatole, with CYP2A19 and CYP2E1 producing the highest amounts of the key metabolite 6-hydroxyskatole that is linked to skatole clearance. The Phase II sulfoconjugation of skatole metabolites is catalysed by sulfotransferase 1A1 (SULT1A1). Levels of 6-OH-3MI-sulfate are negatively correlated with skatole levels in fat, while levels of the metabolites HMOI and I3C are positively correlated with skatole levels in fat. Thus, pigs with high levels of the liver enzymes that produce 6-OH-3MI-sulfate will be able to metabolize skatole and clear it from the body. Pigs with low levels of these enzymes can have high levels of skatole in the fat, due to insufficient capacity of the liver to convert skatole to these key metabolites. This becomes a problem if the amount of skatole absorbed from the gut or the environment is high. The activity of the skatole metabolizing enzymes is increased in castrates compared with entire males, due to the inhibition of these enzymes by sex hormones. This explains why high skatole concentrations in the adipose tissue are mainly found in some entire males and not in castrates.

Several nuclear receptors have been implicated in the co-regulation of androstenone and skatole

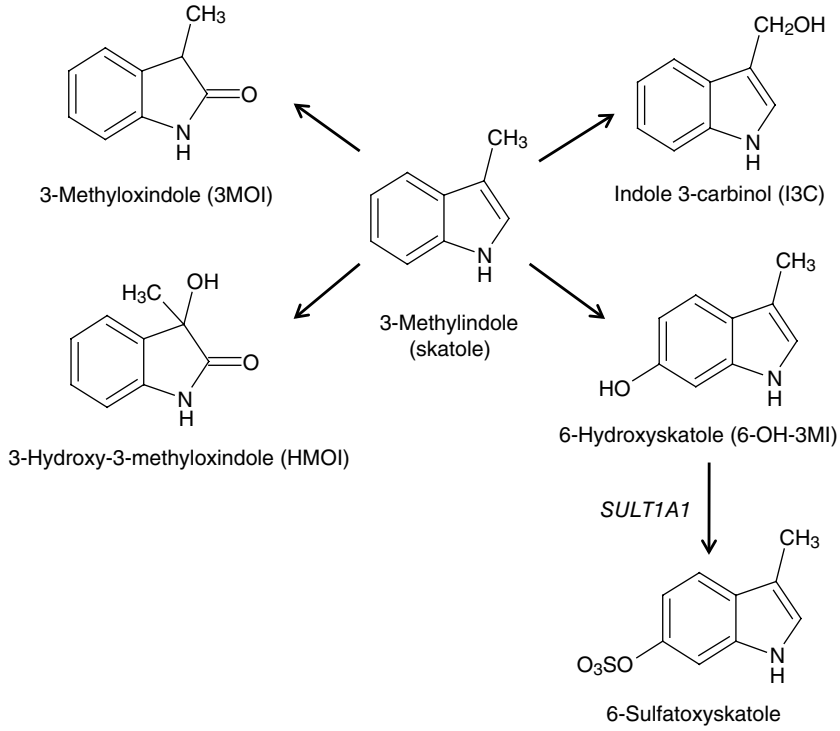


Fig. 3.13. Hepatic metabolism of skatole.

metabolism. These include the constitutive androstane receptor (CAR), pregnane X receptor (PXR) and farnesoid X receptor (FXR) as well as the transcription factors COUP-TF1 and HNF-1. Activation of CAR, PXR or FXR increased the metabolism of skatole, while activation of PXR dramatically increased androstenone metabolism in hepatocytes. Some of the metabolites of androstenone and skatole have been shown to affect the activity of these nuclear receptors and thereby affect the expression of genes involved in the metabolism of androstenone and skatole. There is also the potential to use various natural products to activate nuclear receptors regulating boar taint metabolism. Steroid hormones such as oestradiol, which are produced in large amounts in male pigs, may also be involved. For more information, see the review by Squires *et al*. (2020).

Potential methods to control boar taint

The extent of boar taint from skatole and androstenone is affected by a number of factors, including diet, management and genetic factors. Efficient methods for controlling boar taint are needed before intact males can be used for pork production. If the incidence of boar taint in intact male pig populations was sufficiently low (less than 5%) and effective detection methods for identifying carcasses with boar taint were available, these carcasses could be sorted out on the slaughter line to be used in processed products.

Diet and management

There is a wide variation among different animals in fat skatole levels at slaughter; this depends on the rate of skatole production, intestinal transit time, intestinal absorption and hepatic metabolism of skatole. Because bacteria in the gut produce skatole, feeding and rearing factors can affect skatole production by altering the gut microbiome or the availability of the substrate tryptophan. Including fermentable carbohydrates, such as inulin, sugar beet pulp and raw potato starch in pig diets decreases the production of skatole by the gut microflora and consequently lowers skatole levels in fat. This may be due to decreased turnover of

cells lining the gut, which act as a source of available tryptophan. Fermentation produces short-chain fatty acids (SCFAs) such as butyrate, which can reduce cell apoptosis and turnover. Fermentable fibre also provides energy for microbial activity, which would decrease the degradation of tryptophan by the bacteria for energy and skatole production and increase the use of tryptophan for bacterial growth. Intestinal transit time and absorption are also affected by diet. Withholding feed on the evening prior to slaughter has been shown to reduce fat skatole levels. Raising pigs on slatted floors decreases skatole levels compared with animals on concrete, probably because the animals are less dirty. Thus, a proper control of the environment and diet may reduce fat skatole levels substantially by decreasing the production and absorption of skatole. Skatole metabolism and clearance is affected by genetic and hormonal factors, as well as SCFAs and metabolites from amino acid metabolism by the gut bacteria. For more information on nutritional strategies to control boar taint, see the review by Bee *et al.* (2020).

Androstenone levels may be reduced by feeding diets to maximize growth rate so that entire males reach market weight before they become sexually mature. However, the timing of puberty can vary by breed and within breed, so this is not a reliable practical method to decrease androstenone levels. Furthermore, a reduction in slaughter weight may not be an economically acceptable approach, since it reduces the amount of meat yield from a carcass. Feeding binding agents to interrupt the enterohepatic circulation of androstenone is a promising method to reduce boar taint from androstenone.

Genetic approaches

Androstenone synthesis increases during puberty, along with increased production of the androgens and oestrogens that are responsible for the better growth performance of intact males. There is a wide variation among different intact males in the accumulation of androstenone in fat. Levels of androstenone also vary among breeds of pigs, with Durocs having dramatically higher levels than Yorkshire, Landrace or Hampshire breeds. The sexual maturity and age of the animal at slaughter weight, as well as the genetic potential for androstenone production and clearance, affect the accumulation of androstenone. Boars can be early maturing with a high potential for androstenone production, late maturing with a high potential for androstenone production, or have a low potential for androstenone production even when sexually mature. The same regulatory systems appear to control the synthesis of all testicular steroids, but the physiological basis for these differences in potential for androstenone production have not been identified. These mechanisms are genetically determined and this explains the high heritability of fat androstenone content, with heritability estimates ranging from 0.25 to 0.87. Heritability estimates for skatole are also moderate to high, so selection for low boar taint should be possible. The production of low-boar-taint lines of pigs using genetic approaches would be a long-term solution to the boar taint problem. However, genetic selection for reduction of androstenone must also be accompanied by control of aggressive and sexual behaviours.

The genetic correlations of androstenone with production traits such as growth rate, feed efficiency or carcass quality are mostly favourable and selection applied in sire lines should tend to decrease boar taint. In dam lines, the genetic relationships of boar taint with reproductive traits differs between breeds, so breeding to reduce boar taint needs to be carefully monitored so that it does not adversely affect reproduction. This was shown in early selection experiments against androstenone, which also decreased the production of androgens and oestrogens and delayed puberty in the gilts of a 'low-androstenone' line.

The identification of genetic markers in genes that specifically affect only boar taint may allow specific selection methods to reduce boar taint to be developed without negative pleiotropic effects on reproduction. The pathways of the synthesis and metabolism of androstenone and skatole have mostly been characterized and the genes involved have been identified. Several quantitative trait loci (QTLs) for boar taint have been found throughout the genome and fine mapping of these loci has also identified important candidate genes. Differentially expressed genes have also been identified using microarray technology in both testis and liver. A number of single nucleotide polymorphisms (SNPs) have been identified in these candidate genes that are associated with decreased levels of boar taint compounds. These markers could be used in future marker-assisted selection programmes for low-boar-taint pigs. For more information, see the review by Larzul (2021).

Another potential genetic approach to control boar taint is using gene editing, which allows the

introduction of specific mutations or novel sequences targeted to a specific place in the genome. The most efficient gene editing tool is the CRISPR/Cas9 system that originated as a defence mechanism of bacteria against foreign viral DNA (see Section 2.4) However, organisms created using gene-editing techniques are classified as genetically modified organisms (GMOs) in some countries, which limits the use and acceptability of gene editing technology.

There are currently two gene-editing approaches under commercial development to control boar taint. These are based on delayed puberty by knocking out the KISSR gene that is involved in the onset of puberty, or inactivating the SRY gene involved in testis development. These strategies will prevent the formation of androstenone but will also reduce the production of the androgens and oestrogens that are responsible for the superior growth performance of boars. An alternative strategy to reduce androstenone synthesis involves editing specific amino acid residues in CYP17A1 and CYB5A that are involved in the synthesis of androstadienol (Squires *et al*., 2019). These residues are in the steroid binding pocket of CYP17A1 and on the surface of CYP17A1 and CYB5A that are involved in binding CYP17A1 to CYB5A. This approach specifically targets the synthesis of androstenone, while not reducing the production of the sex steroids that are responsible for the male phenotype and superior growth performance of males.

Immunological methods

A number of immunological approaches could potentially be used to reduce boar taint from androstenone. Attempts have been made to remove 5α-androstenone by active immunization against the steroid, but it was still deposited in the body tissues. The removal of the 16-androstene steroids should not adversely affect growth performance, since they show little or no anabolic activity.

Castrating pigs at 2 or 3 weeks before slaughter reduces androstenone content in fat to levels similar to those in castrates and gilts. However, surgical castration of older animals cannot be used in practice. Interfering with testicular function can also reduce boar taint. Antagonists or agonists for GnRH can be used to interfere with gonadotrophin production.

Another approach is the immunocastration of male pigs by immunizing against GnRH a few weeks prior to slaughter. This yields the anabolic effects of the androgens early in life but shuts down all steroid biosynthesis before slaughter, which allows any androstenone to be cleared from the fat stores. Immunocastration also reduces the size of reproductive organs, decreases sperm numbers and controls aggressive behavior. Fat skatole content is also reduced, due to enhanced metabolic clearance by the liver after steroid production is reduced, as occurs in surgically castrated pigs. IGF-1 concentrations in plasma were also reduced in immunocastrated pigs, which might affect skatole production by decreasing the turnover of mucosal cells in the ileum and colon.

An anti-GnRH vaccine for immunocastration from Zoetis (named Improvac, Improvest, Innosure and Vivax), developed in Australia, has been used for a long time in Australia and New Zealand. It was approved for use in the EU in July 2009 but its use in Europe has been significant only in Belgium. Immunocastration has met with more success in Central and South America, particularly in Brazil. More recently, CEVA developed its Valora vaccine, which is registered for commercial use in Mexico and other countries in South America.

Two doses of vaccine (2 ml of 200 μg ml^{-1} GnRH conjugate) are given at an interval of at least 4 weeks. The second dose should be given 4–5 weeks before slaughter and a third vaccination is needed for pigs slaughtered at a much heavier weight. The vaccine is administered at the base of the ear with a special vaccinator designed to prevent accidental needle sticks. Workers accidentally injected with the vaccine may also raise antibodies against GnRH and potentially become sterile, but this is not permanent. However, the main concern about immunocastration is the acceptability of pork from immunocastrated pigs by the general public.

The growth performance of immunocastrates is closer to entire males than surgical castrates, with more rapid growth, lower feed intake and more efficient feed conversion of immunocastrates compared with surgical castrates. This is attributable to the positive effects of androgens secreted from the testes prior to immunization. It has also been suggested to treat immunocastrates with β-agonists (Paylean®, see Section 3.7) to improve performance following the second vaccination. Compared with intact males, immunocastrated pigs have a higher fat content in their carcass and increased feed intake, which may be due to decreased levels of oestrogen. An economic model to assess the costs and returns of using Improvac in the US market suggests an additional income of $5.48/pig for

immunocastrates compared with surgical castration. For more information on immunocastration, see Batorek *et al.* (2012); for more information on the economic analysis of castration, see Bee *et al.* (2015).

3.4 Somatotrophin

Key concepts

- Somatotrophin or growth hormone (ST, GH) is a peptide hormone that is produced by the anterior pituitary in response to GH-releasing hormone (GHRH) and somatostatin.
- Ghrelin is a peptide that binds to the growth hormone secretagogue receptor (GHS-R) to stimulate pulsatile GHRH and ST release.
- ST has direct anabolic effects to increase protein synthesis and decrease lipid accumulation.
- The indirect effects of ST on cell growth are mediated via insulin-like growth factor-1 (IGF-1), which is produced primarily in the liver.

Somatotrophin or growth hormone (ST, GH) is the most important peptide hormone affecting growth. Experiments in the 1930s demonstrated that rats injected with alkaline extracts of pituitary gland gained more weight and had more muscle and less fat than controls. In 1945, growth hormone was isolated from anterior pituitary and experiments evaluating the effects of crude preparations of porcine somatotrophin (pST) began in pigs. However, prior to the 1980s, studies with domestic livestock and commercial application of ST were limited because ST had to be extracted from pituitary glands of slaughtered animals. Thus, there was a very limited supply of ST, and what was available varied greatly in quality. Subsequently, advances in recombinant DNA technology made it possible to produce large quantities of high-purity ST (see Section 2.3).

Somatotrophins from domestic livestock contain 191 amino acids and there is a high degree of sequence homology between STs from different species. There are 18 differences in amino acid sequence between pST and bovine ST (bST), but only 2 differences between bST and ovine ST (oST). Chicken ST (cST) has only 77% sequence identity to bST. There are considerable sequence differences between human ST (hST) and bST or pST, so pituitary preparations from farm animals are not active in humans. Variants of ST are produced by alternative splicing of the mRNA transcript in the pituitary gland, and four major variants of bST have been identified. ST has four α-helices, nonhelical chains, two disulfide bridges and a hydrophobic core. Two ST receptor binding sites are located on opposite sides of a bundle of α-helices.

Applications of ST

Treatment of pigs with pST increases protein accretion and decreases fat deposition in boars, barrows and gilts in both poor and improved genotypes. The response to pST can vary according to initial body weight, length of treatment, breed, sex, dose of pST and diet composition. Maximally effective doses can increase average daily gain by 10–20%, improve feed efficiency by 15–30%, decrease feed intake by 10–15%, decrease lipid accretion by 70% and increase protein deposition by 50%. A daily dose of 30–69 μg kg^{-1} body weight given between 50 kg and slaughter improves growth rate, feed conversion efficiency and lean content by 10–22%, while decreasing carcass fat by 30%. Since pST stimulates protein deposition in all tissues, a slight decrease in dressing percentage may be found, due to an increase in weight of the internal organs. There may be small negative effects on meat tenderness from pST treatment, but no effect on juiciness. However, treatment of pigs with pST has been reported to reduce chondrocyte metabolism and compromise cartilage, bone and joint development in growing animals.

Bovine ST is less effective in growing cattle than pST is in pigs. Daily gain, feed efficiency and carcass lean content in growing cattle are improved by 12%, 9% and 5%, respectively, and in sheep by 18%, 14% and 10%, respectively, by bST treatment. Carcass fat content was decreased by 15% in sheep and cattle by bST treatment. The lipolytic effects of ST contribute to improved feed efficiency while maintaining growth rate. As a result, feed costs are lower and carcass grade is improved.

Results with chickens have been mixed, but this may be because of the mode of delivery. Episodic administration of cST to older birds markedly improved growth performance, while continuous infusion had no effect. ST is largely ineffective in promoting growth or altering metabolism during the early post-hatch period, when the bird is already growing rapidly and the rate of fat deposition is low. For more information on factors affecting growth in birds, see Saxena *et al.* (2009).

Bovine ST has dramatic effects on milk production and is approved for use in the dairy industry in the USA. This aspect is discussed in Section 4.1.

For interest

A mutation in the growth hormone receptor in dwarf (*dw*/*dw*) chickens reduces body weight to 30–40% of normal birds.

Control of ST release

ST release from the anterior pituitary gland is regulated by hormones produced in the hypothalamus (Fig. 3.14). ST release is stimulated by growth hormone-releasing hormone (GHRH) and decreased by somatostatin (SS). ST release occurs in a pulsatile pattern with peaks higher at night and this pattern is regulated by the feedback of ST on paraventricular neurons in the hypothalamus to cause the release of SS. The production of SS suppresses the activity of the GHRH neurons in the arcuate nucleus and subsequently decreases the release of ST by the pituitary. The decrease in ST then reduces the negative feedback on the hypothalamus, which increases GHRH production to increase ST release from the pituitary. This cycle of events results in a pulsatile release of ST. For more information, see the review by Ranke and Wit (2018).

Somatostatin exists as both 14 amino acid (SS-14) and 28 amino acid (SS-28) forms that interact with different receptors. SS-14 is the predominant form and has the same sequence in different species. Somatostatin is expressed in brain and in specialized D cells in the gastrointestinal tract and pancreatic islets. It acts to inhibit the secretion of most hormones and, in addition to its endocrine effects, can act locally as a paracrine or autocrine regulator. In the stomach, SS inhibits the release of gastrin and hydrochloric acid, while in the pancreatic islets, SS regulates the secretion of glucagon and insulin. Androgens stimulate while oestrogens decrease the production of SS in the brain; this may in part explain the differences in ST secretion patterns between males and females. Octapeptide analogues of SS are octreotide (Sandostatin®, Novartis Pharmaceuticals) (Fig. 3.15), lanreotide and vapreotide, and these are used for the treatment of agromegaly and to decrease gastric secretions.

There are five major forms of SS receptors, which vary in their tissue distribution. SS receptors are coupled to several populations of K^+ channels and their activation causes hyperpolarization of the cell membranes, leading to decreased action potential and decreased intracellular calcium.

GHRH was first isolated from pancreatic islet tumours. It is a 44 amino acid peptide that is part of a family of brain–gut peptides that includes glucagon, glucagon-like peptide I, vasoactive intestinal polypeptide, secretin and gastric inhibitory peptide (see Section 3.10). In addition to regulating secretion of ST, GHRH has been implicated in promoting sleep and increasing appetite, particularly for protein. ST secretion also increases during rapid eye movement (REM) sleep.

Pulsatile GHRH release can be stimulated by activation of the growth hormone secretagogue receptor (GHS-R) in the arcuate nucleus of the hypothalamus. The GHS-R is expressed in the hypothalamus as well as in the pituitary and peripheral tissues. The GHS-R is not activated by GHRH or somatostatin but was first identified by binding to met-enkephalin. Based on this, a number of peptide analogues of leu- and met-enkephalins

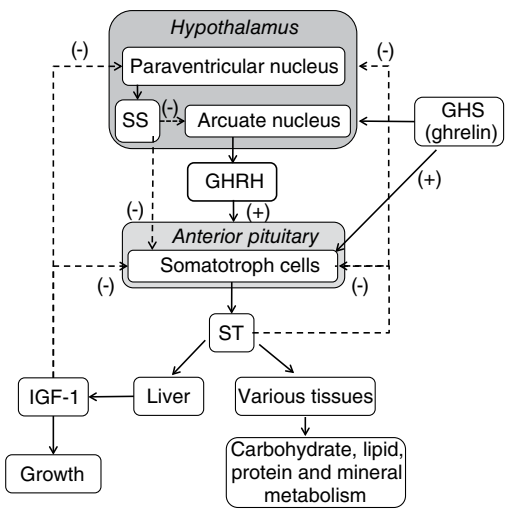


Fig. 3.14. Regulation of growth hormone (ST) release. SS, somatostatin; GHS, growth hormone secretagogue.

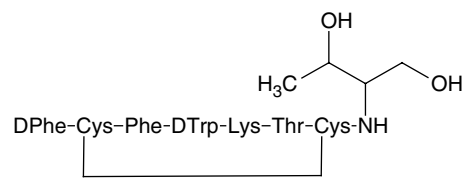


Fig. 3.15. Structure of octreotide, an analogue of somatostatin.

known as growth hormone-releasing peptides (GHRPs) have been identified. GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) was one of the first analogues developed as a potent stimulator of pulsatile ST secretion, but the GHRPs had low oral bioavailability of about 0.3%. Other non-peptide derivatives were developed (Fig. 3.16) and L-692,429 was the first of these, with 4% bioavailability in humans. Later, the spiropiperidine MK-0677 (ibutamoren) was developed to have good bioavailability and longer half-life, being effective for 24 h. The benzolactam L-739,943 had similar biological and pharmacokinetic properties but different structure from MK-0677. The benzylpiperidine L-163,540 (AdisInsight) has a shorter half-life and is used for finer control over ST and IGF-1 levels.

Ghrelin is a 28 amino acid peptide that was subsequently identified as the natural ligand of the GHS-R stimulating GHRH release. The biologically active form of ghrelin is acetylated by the enzyme ghrelin-*O*-acyltransferase at serine-3 with an *n*-octanoic acid side group that is necessary for biological activity. There are two types of GHS receptors, GHS-R1a and GHS-R1b, which differ in the total number of amino acids and the number of trans-membrane domains. The GHS-R1a is primarily bound and stimulated by activated ghrelin. Ghrelin is produced in the stomach, kidney and placenta, and its primary role is the regulation of appetite and energy balance (see Section 3.10). Intracerebroventricular injections of ghrelin stimulated feeding and weight gain in rats. Ghrelin blocked the action of leptin in reducing feed intake, suggesting that there is a competitive interaction between ghrelin and leptin in regulating feed intake. Ghrelin also regulates gastric acid secretion by activating the vagus system. Ghrelin and leptin form an essential link between the gut, energy homeostasis and regulation of ST and gonadotrophins. For more information on growth hormone secretagogues, see Ishida *et al*. (2020).

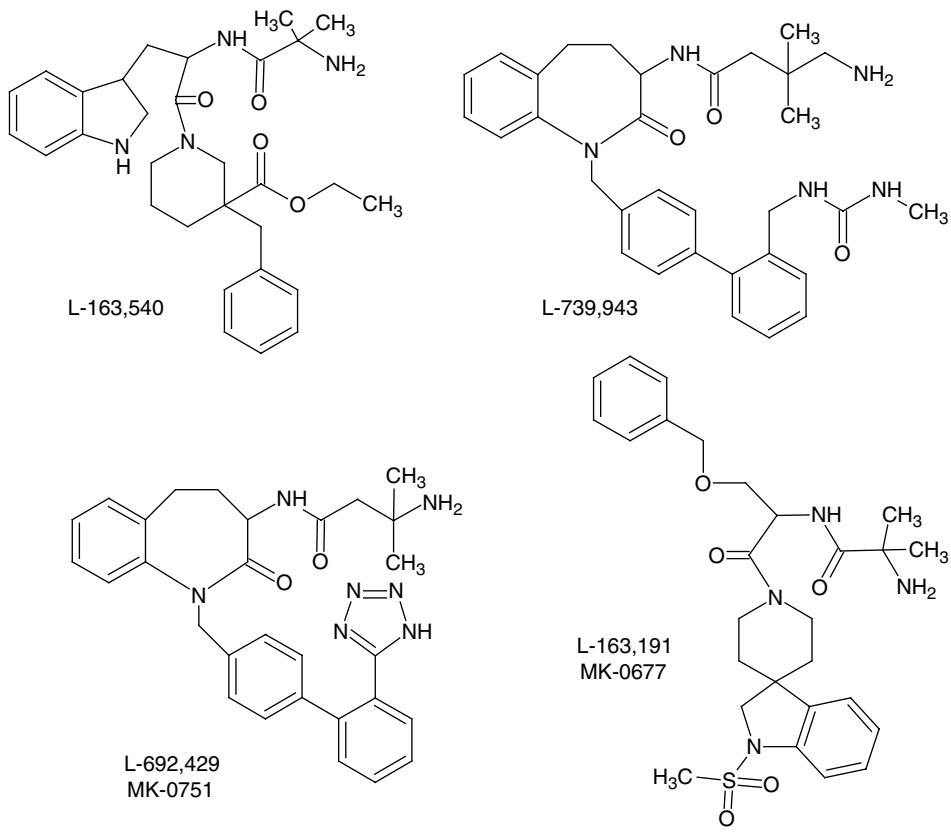


Fig. 3.16. Growth hormone secretagogues.

Binding of GHRH to its receptors on pituitary somatotrophs increases intracellular levels of cyclic adenosine monophosphate (cAMP). Somatostatin binds to subtype-2 receptors and hyperpolarizes somatotroph membranes to inhibit ST release. The GHS-R is a G protein-coupled receptor which acts to activate the Gαq and Gαs subunits and both the PLC and cAMP pathway (CREB-P) (see Section 1.3). The presence of GHRP-6 along with GHRH potentiates the increase in cAMP due to GHRH alone, indicating that there is some interaction between the different signal transduction pathways. Co-administration of GHRH and ghrelin gives a synergistic effect on ST secretion.

Plasma levels of ST and the pulse amplitude for ST are higher in women than in men, but the pulse frequency is similar in males and females (Fig. 3.17). The amplitude and number of oscillations of ST release decline during ageing. Decreased levels of ST are associated with a reduced nitrogen balance and a decline in body condition and muscle tone. The decrease in ST may be due to a decrease in the endogenous ligand(s) of the GHS-R (such as ghrelin) during ageing. Chronic oral administration of MK-0677 once daily to dogs produces a sustained physiological pattern of pulsatile ST release. However, stimulation of ST release by isolated pituitary cells *in vitro* is not sustained, with desensitization occurring within a few minutes. Administration of L-dopa to old rats also restores the amplitude of ST release to what is seen in young animals. It has been suggested that GHS-R ligands may modulate dopamine release from hypothalamic neurons to affect pulsatile release of ST.

Mechanism of action of ST

Somatotrophin affects a wide range of somatogenic and metabolic processes to increase lean tissue growth. Somatotrophin has two distinct types of effects: direct and indirect. The direct metabolic effects are due to ST binding to receptors on target cells and these include effects on carbohydrate, lipid, protein and mineral metabolism. The indirect somatogenic effects of ST are those related to cell proliferation and these are mediated primarily by insulin-like growth factors (IGF-1 and IGF-2, somatomedins), which are potent mitogens with some sequence similarities to insulin. IGF-2 is a major fetal growth factor, while IGF-1 is a major growth factor in adults (Fig. 3.18).

Direct effects

ST RECEPTORS The biological actions of ST begin with ST binding to its receptor on the cell surface. The ST receptor is a transmembrane protein of 634–638 amino acids with a molecular mass of

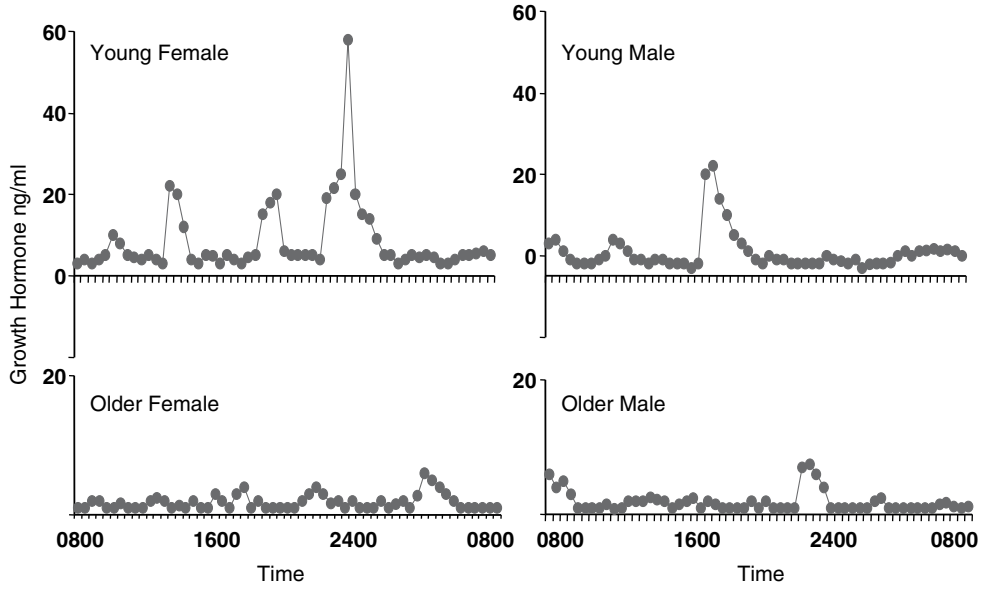


Fig. 3.17. Age and gender effects on the pattern of growth hormone secretion (redrawn from Smith *et al*., 1996).

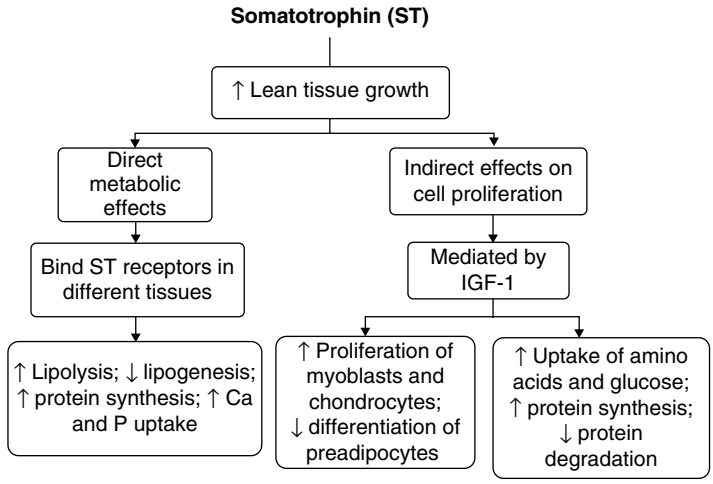


Fig. 3.18. Effects of somatotrophin on growth and metabolism.

about 70 kDa. The free receptor exists in the cell membrane as an inactive dimer and is converted to an activated receptor complex by a conformational change when bound to a single ST molecule. The ST receptor is a member of a superfamily of cytokine receptors, which includes prolactin, erythropoietin, interferons and interleukins (see Section 1.3). The activated ST receptor has bound Janus kinase 2, which phosphorylates STAT proteins (particularly STAT5b), which promote transcription resulting in the generation of IGF-1. This also results in proliferative effects through the MAPK system (see Section 1.3). The metabolic effects of ST are due to the activation of the insulin receptor substrate (IRS) and phosphatidylinositol 3′ kinase (PI3K) and do not depend on IGF-1.

A growth hormone-binding protein (GHBP), which is essentially the extracellular domain of the ST receptor that is also produced from the ST receptor gene, is present in plasma of many species. GHBP enhances the growth-promoting effects of ST, probably by increasing the half-life of ST in the circulation.

METABOLIC EFFECTS The metabolic effects of ST in different tissues are summarized in Table 3.3. There is a dramatic decrease in lipid accretion in ST-treated animals. This allows more nutrients, such as glucose, to be available for increased growth of lean tissue and improves commercial production efficiency by reducing the proportion of nutrients used for the synthesis of body fat. The effects of ST on lipid metabolism are chronic rather than acute. When animals are in positive energy balance, ST reduces the rate of lipogenesis, while lipolysis is increased in animals under negative energy balance. In growing pigs, ST decreases the uptake of glucose for lipid synthesis by adipocytes through decreased synthesis of the glucose transporter protein GLUT4. ST also decreases the synthesis of lipogenic enzymes, including fatty acid synthase and acetyl-CoA carboxylase. ST also decreases the stimulatory effects of insulin on glucose uptake and utilization by adipocytes and increases the lipolytic responses to epinephrine. ST treatment of pigs

Table 3.3. Summary of metabolic effects of somatotrophin in different tissues.

Tissue type	Metabolic effect
Skeletal muscle	Increased protein synthesis/ accretion
Adipose tissue	Increased lipolysis, decreased glucose uptake and lipogenesis
Liver	Increased glucose output via glycogenolysis and gluconeogenesis, regulation of CYP genes
Intestine	Increased calcium and phosphorus uptake
Mammary gland	Increased milk synthesis by increased uptake of nutrients and increased blood flow
Various tissues	Decreased amino acid and glucose oxidation; increased oxidation of free fatty acids; decreased effects of insulin

decreases glucose utilization by adipose tissue from 40% to 7% of the whole-body glucose turnover.

The protein anabolic effect of ST occurs almost exclusively by increasing protein synthesis, with no measurable effects on protein catabolism. Alterations in tissue response to other endocrine factors, such as insulin, may also be important. Sufficient levels of plasma amino acids are needed to sustain the increased level of protein synthesis. Maintenance energy requirements are also 10–20% higher in pST-treated pigs, because of increased protein synthesis in muscle and in visceral tissues such as liver and gut.

In growing pigs, pST treatment increases the apparent efficiency of use of dietary protein for protein deposition by 25–50%, depending on growth stage, quality of dietary protein and the sex of the pig. Young pigs that are less than about 40 days of age have little response to pST treatment, since they are already growing rapidly with a high efficiency of utilization of dietary protein. The rate of protein deposition is increased by 16%, 25% and 74% in gilts and barrows in the weight ranges of 10–20 kg, 20–55 kg and 55–100 kg, respectively. There is no effect of pST on protein digestibility and only a marginal increase in the dietary protein requirement for 25–60 kg pigs. Increases in protein deposition from pST treatment are greater in finisher pigs weighing 60–90 kg and this increases the dietary protein requirement. This is especially important in boars of improved genotypes that already have a high efficiency of use of dietary protein. Thus, pST acts by separately increasing the efficiency of amino acid utilization and the maximal rate of protein accretion. Decreased levels of plasma urea nitrogen and the activity of lysine α-ketoglutarate reductase are seen following ST treatment, which suggests that oxidation of amino acids is reduced.

Indirect effects

The major role of ST in stimulating growth is mediated via IGF-1 (see also Section 4.1). High blood levels of IGF-1 decrease the secretion of ST by direct negative feedback action on the pituitary and also by stimulating SS production by the hypothalamus (see Fig 3.14).

IGF-1 is a single polypeptide chain of about 7.5 kDa and the sequences of human, porcine and bovine IGF-1 are identical. There are at least six binding proteins for IGF-1 and almost all of the IGF-1 is bound to insulin-like growth factor binding proteins (IGFBPs). These IGFBPs may protect IGF-1 from degradation by proteases, potentiate or inhibit the interactions of IGF-1 with its target tissues, or have IGF-1-independent actions. IGF-1 is synthesized primarily in the liver, along with its major binding protein, IGFBP3, and the third member of the ternary binding complex, the acid labile subunit (ALS). IGF-1 is liberated from the ternary complex in the circulation by cleavage of IGFBP by the protease pappalysin, which frees IGF-1 to pass through the capillary epithelium. Local synthesis of IGF-1 in muscle and adipose tissue is also important. IGF-1 interacts with IGFR1 and IGFR2 receptors, which differ in structure, specificity and signalling mechanism. IGFR1 is similar to the insulin receptor, while IGFR2 is a monomeric protein that has weak affinity for IGF-1.

ST has a major impact on muscle fibre development through production of IGF-1 and 2. In addition to systemic IGF-1 produced in the liver, IGF-1 is also produced locally in the muscle, where it has autocrine and paracrine effects to stimulate satellite cell proliferation (see Section 3.2 on the indirect effects of anabolic steroids). The IGFs are involved initially in regulating the proliferation of myoblasts and then their subsequent differentiation into myotubes. The differentiation process occurs primarily through the type I IGF receptor. IGF-1 also stimulates amino acid and glucose uptake and protein synthesis and decreases the rate of protein degradation in muscle, and these effects may be mediated through the insulin receptor.

The expression of the different IGFBPs varies during myogenic differentiation and it is thought that they inhibit the effects of IGFs. This was demonstrated using the IGF analogue des(1-3) IGF-1, which has a reduced affinity for IGFBP but binds the type I receptor with similar affinity as native IGF-1. Experiments with muscle cell lines showed that des(1-3) IGF-1 was more potent in stimulating cell proliferation and differentiation than native IGF-1. In addition, transgenic mice that overexpress IGFBP-2 had a lower body weight gain than control mice.

Adipose tissue has receptors for ST and produces both IGF-1 and IGFBPs. In tissue culture, ST decreases the differentiation of preadipocytes into adipocytes and inhibits the expression of fatty acid synthase and hormone-sensitive lipase. ST also reduces the volume of mature adipocytes to reduce body fat. IGF-1 also stimulates the proliferation of chondrocytes or cartilage cells, to increase bone growth, which can reduce the carcass dressing percentage.

Although some of the effects of pST are mediated by IGF-1, treatment of finisher pigs with IGF-1 does not affect growth, possibly due to the rapid turnover of IGF-1. Treatment of finisher pigs with the potent synthetic analogue LR3 IGF-1 reduces growth rate, the amplitude of endogenous pST pulsatile release and plasma levels of IGF-1 and its major plasma-binding protein. Thus, negative feedback limits the effectiveness of IGF-1 treatment in finisher pigs. In neonatal pigs, LR3 IGF-1 infusion increases growth of the gut and visceral tissues, particularly in pigs under nutritional stress.

Peptide growth factors may be orally active in the neonatal pig and could potentially be added to diets of early-weaned or supplemental-fed piglets to improve health and growth rate.

For interest

The IGF-1 gene locus affects the body size of dogs. This is due to a single (C/T) variant in a long non-coding antisense RNA (IGF1-AS) that interacts with the IGF1 mRNA, creating a duplex that affects translation of the mRNA (Plassais *et al*., 2022).

Delivery/dose effects

Porcine ST was approved in 1994 for use in pigs in Australia, using daily injections of 3–5 mg per pig for finishing pigs, starting at 35 days before slaughter. It has been approved for use in 14 countries but has not been approved for use in North America and it is banned in the EU. ST is not orally active and most studies have used daily injection as a practical means of delivery of pST. The ADG and rates of protein and ash accretion are maximally stimulated at a daily pST dose of about 100 μg kg^{-1} BW. However, rates of lipid accretion and feed:gain ratio decrease in a more linear manner up to a dose of 200 μg kg^{-1} BW. Sustained use of pST results in osteochondrosis, causing severe discomfort in the joints of treated pigs. For more information, see Dunshea *et al*. (2016).

The most investigated formulation for pST has been the implant, with the goal of a one-time treatment that provides pST for the entire finishing period. Sustained-release implants have been designed to deliver approximately 2 mg of pST per day for 42 days. The implants consist of recombinant pST having an *N*-alanyl residue linked to the natural pST sequence in a solution of 49.5% 1 M sodium phosphate, 49.5% glycerol and 1% Tween 20. Pigs treated with these implants had increased efficiency of gain through decreased feed consumption but the rate of gain was not increased. It appears that the large peaks in pST caused by daily injection are necessary to activate the physiological processes related to growth.

Administration of ST to rats by constant infusion produces a male pattern of response in liver enzymes, while a pulsatile delivery elicits a female response. In chickens, the pulse amplitude of ST in plasma is high at early ages during rapid growth and decreases at older ages when growth rate is decreased.

Growth hormone secretagogues (GHSs) such as MK-0677 have been used in humans as an alternative to injectable ST. They have the advantage of oral dosing and producing a pulsatile, physiological ST profile. However, the GHS-R pathway is subject to negative feedback, so the sustained supraphysiological levels of ST that can be obtained with ST or GHRH injection are not possible with GHS treatment.

A slow-release oil-based formulation of zinc methionyl bST (Sometribove®) is available, which improved growth performance and increased carcass leanness in lambs when administered weekly or once every 2 weeks. This is also used to improve milk yield in dairy cattle in the USA (Poslilac®, from Monsanto) (see Section 4.1). Equine ST (methionyl eST, EquiGen®) is also used as an aid to improve nitrogen retention in aged horses (at least 15 years of age). It has been shown to decrease plasma urea nitrogen and urinary nitrogen, indicating that it increased protein synthesis. It is administered for 42 days, starting at 10 μg kg^{-1} BW daily for the first week and then increasing to 20 μg kg^{-1} BW daily for weeks 2–6. ST is given by deep muscular injection, rotating the site from the neck, pectoral muscle and rump. Recombinant bST has also been shown to increase growth rate and improve feed efficiency and protein deposition in both young and older salmonid fish.

Immunization against somatostatin has also been used to increase the secretion of ST. Some work on developing anti-idiotypic antibodies for ST has also been done (see Section 2.4). Transgenic animals with ST genes (pigs, fish) have also been generated.

Safety/quality aspects

ST is a natural protein that is destroyed by cooking and rapidly broken down in the gut, so the potential threat from residues should be small. A slight

reduction in meat tenderness in pigs treated with pST may be due to decreased intramuscular fat content.

3.5 Adipokines

Key concepts

- White adipose tissue secretes a number of adipokines that regulate energy metabolism, reproduction and immune function.
- Leptin is secreted in proportion to the amount of adipose tissue to decrease appetite, reduce lipogenesis and increase lipid oxidation, increase energy utilization and improve reproduction.
- Adiponectin secretion is decreased with increases in adipose tissue to inhibit energy expenditure, increase feed intake and stimulate free fatty acid utilization.
- Both leptin and adiponectin function via the key metabolic energy sensor AMP-activated protein kinase (AMPK).
- Irisin is produced by muscle and white adipose tissue and stimulates the browning of white adipose tissue.
- Inflammatory cytokines produced by macrophages and adipocytes, and resistin produced by leukocytes and adipocytes, antagonize the anabolic effects of insulin and promote inflammatory responses.

White adipose tissue is a complex endocrine organ, as well as an energy storage depot and form of insulation. Adipose tissue secretes a number of endocrine factors known as adipokines, which regulate energy metabolism, cardiovascular function, reproductive status and immune function (Fig. 3.19). Adipokines act in a paracrine, autocrine and endocrine fashion and include hormonal and growth factors, cytokines and chemokines, which can modulate the actions of insulin and lipid and glucose metabolism. The main adipokines include leptin, adiponectin, irisin and resistin, along with cytokines including tumour-necrosis factor, interleukin 6, chemokine (C–C motif) ligand 2, interleukin 10 and transforming growth factor-β. Adipose tissue consists primarily of adipocytes, but it also contains a number of other cell types, including preadipocytes, stem cells, endothelial cells, pericytes, monocytes/macrophages and nerve cells. Adipocytes from different anatomical locations may vary due to local effects on differentiation and gene expression and secrete different patterns of adipokines. In obese individuals, increased production of adipokines and cytokines can contribute to diabetes, cardiovascular disease and cancer. For more information, see Recinella *et al.* (2020).

Leptin

Leptin is a 16 kDa protein hormone produced primarily by white adipose tissue and is the product of the *lep* (also known as *obese* (*ob*)) gene. It is structurally similar to the long-chain helical cytokines, such as interleukin-6 (IL-6) and granulocyte-colony stimulating factor (G-CSF). Both the nucleotide and amino acid sequences are highly conserved in vertebrate species. Leptin secretion is higher in subcutaneous than omental adipocytes. Leptin is also expressed at lower levels in placenta, stomach, mammary epithelium and skeletal muscle. Leptin is thought to function as an 'adipostat' and long-term regulator of energy reserves in the form of adipose tissue in the animal. When there is a lot of adipose tissue, there is increased production of leptin, which enters the bloodstream, passes through the blood–brain barrier and activates the satiety centres in the hypothalamus to reduce food intake. Conversely, when adipose tissue reserves decrease due to limited availability of food, leptin levels decrease and appetite increases. Leptin is thus an important regulator of appetite, whole-body energy balance and body composition (see Section 3.10). Leptin is also important in metabolic adaptation to starvation by regulating metabolic rate and improving reproductive and thyroid function (Fig. 3.19).

Plasma leptin varies in a pulsatile manner, with approximately 30 pulses in 24 h in humans. There is also a diurnal rhythm in leptin levels, with highest levels at 01.00 hours and lowest levels at 11.00 hours, but varying the timing of meals can alter this rhythm.

Leptin is mutated and inactive in the homozygous obese (*ob/ob*) mouse; leptin treatment of these mice reduces feed intake and body weight by about 40% after 33 days of treatment. Pair-fed (*ob/ob*) mice that are not treated with leptin lose significantly less weight than those treated with leptin. Body weight loss from leptin treatment is due almost exclusively to loss of adipose tissue, with lean mass unaffected. Leptin treatment of normal wild-type mice also reduces feed intake and body fat.

Up to six different serum-binding proteins for leptin have been identified and leptin circulates in the blood in both free and protein-bound forms.

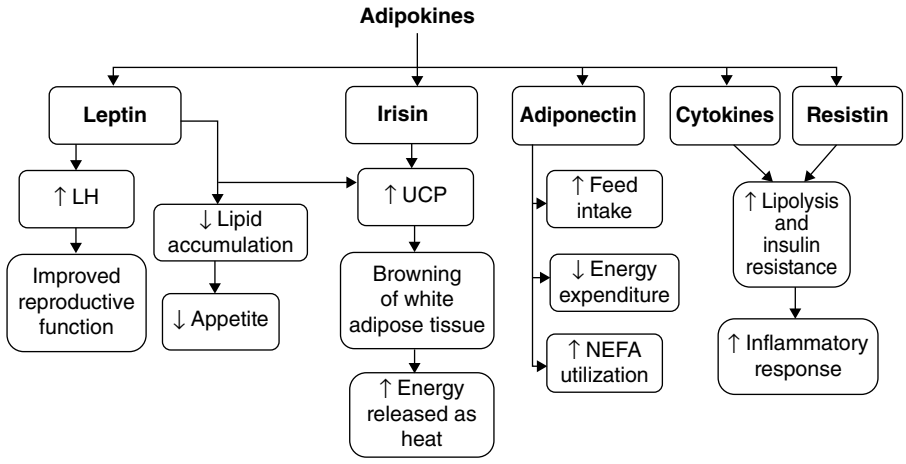


Fig. 3.19. Metabolic effects of adipokines. UCP, uncoupling protein; NEFA, non-esterified fatty acid.

The free form of leptin is the biologically active form and the equilibrium between free and bound leptin regulates leptin bioavailability. The amount of the binding proteins is reduced and the percentage of free and unbound leptin is increased in obesity. The kidney is the major site of leptin metabolism and clearance.

Leptin receptors

Leptin receptors (Lep-R or Ob-R) are located in the hypothalamus as well as on peripheral target tissues. Spontaneous mutations in the leptin receptors in *db/db* mice and *fa/fa* rats result in inactive leptin receptors and severe obesity that is resistant to leptin treatment. The receptor has been cloned from a variety of tissues and is part of the class I cytokine receptor family, which includes growth hormone, prolactin, interleukin-6 and leukaemia inhibitory factor. The receptor exists in multiple forms with a common extracellular sequence and a variable-length cytoplasmic domain. Alternate splicing of the mRNA from a single gene produces the six isoforms of the leptin receptor (Lep-Ra, b, c, d, e and f). The receptor does not have an intrinsic tyrosine kinase activity but acts by association with cytoplasmic JAK (see Section 1.3), which phosphorylates and activates STAT proteins. Since tyrosine 1138 is required for binding and activation of STAT3, shorter isoforms of the receptor missing this residue cannot signal via STAT proteins.

The short forms of the receptor (Lep-Ra, c, d and f) are thought to act as transport proteins to allow leptin to cross the blood–brain barrier or for renal clearance. Leptin resistance may be caused by decreased leptin transport across the blood–brain barrier or insensitivity of the hypothalamus to leptin. The soluble form, Lep-Re, acts as a binding protein for leptin in the blood. A short form of the leptin receptors in the adrenal medulla may be involved in epinephrine secretion. The long form of the leptin receptor (Lep-Rb) is found in areas of the hypothalamus that are involved in the regulation of appetite and body weight, namely the arcuate, dorsomedial, ventromedial and paraventricular nuclei. The effects of leptin on food intake are mediated by inhibiting the actions of orexigenic neurons in the arcuate nucleus that express neuropeptide Y (NPY) and agouti-related protein (AgRP), and stimulating the anorexigenic neurons that express pro-opiomelanocortin (POMC), a precursor of α-melanocyte-stimulating hormone (αMSH), and cocaine- and amphetamine-related transcript (CART) (see Section 3.10). These neurons project to other areas of the hypothalamus to suppress feeding, stimulate thermogenesis and lipid oxidation via sympathetic nerve stimulation of β3-adrenergic receptors in brown fat, and enhance insulin sensitivity in peripheral organs. For more information, see the review by Wauman *et al*. (2017).

Involvement in energy metabolism and reproduction

Leptin signalling is the most crucial factor that controls body weight by balancing food intake and

energy expenditure to adapt to altered energy states like fasting or starvation. Leptin treatment of *ob/ob* mice that have a mutation in the leptin gene and are unable to produce leptin decreases serum insulin and glucose and increases oxygen consumption, locomotor activity and body temperature to that found in normal wild-type mice. Leptin is more effective in the *ob/ob* mice than in normal animals and even the highest dose did not cause metabolic indices to exceed normal levels. These effects may be mediated by the sympathetic nervous system, through the effects of norepinephrine on brown adipose tissue. Catecholamines and β-agonists decrease leptin levels in the long term, while insulin and glucocorticoids act synergistically to increase leptin levels over the long term. Leptin levels are also increased by high-fat diets. Production of leptin is increased by proinflammatory cytokines such as TNFα, interleukin-1β and IL-6.

Leptin also causes local tissue effects that are mediated by mitochondrial uncoupling proteins (UCPs). It stimulates synthesis of UCP mRNA and protein in muscle and adipose tissue. The UCPs increase proton leakage through the inner mitochondrial membrane and thus uncouple oxidative phosphorylation and adenosine triphosphate (ATP) synthesis from mitochondrial electron transport, with the energy released as heat.

Females with poor body condition do not reproduce well and leptin may act to signal the reproductive system that sufficient body fat is present to support a pregnancy. Leptin treatment increases sexual development in both male and female *ob/ob* mice, resulting in increased serum luteinizing hormone (LH) and ovary and uterine weights in females, and increased serum follicle-stimulating hormone (FSH), testes and seminal vesicle weights and sperm counts in males. Levels of leptin are two to four times higher in women than in men, and leptin levels increase near puberty in girls but not in boys. Leptin production is decreased by androgens (testosterone and dihydrotestosterone) but increased by oestrogens.

In seasonal breeders such as sheep, which have seasonal cycles of body weight and reproduction that are driven by photoperiod, the sensitivity of the hypothalamus to leptin varies with the season. During long days, when they have increased appetite and body weight, the hypothalamus is insensitive to high leptin levels. However, during short day length, appetite and body weight are decreased in response to leptin. There is an inverse relationship between leptin and melatonin production.

Leptin levels vary during fetal development and are correlated with fetal size. Leptin may be involved in signalling nutrient availability and in regulating growth and development of the fetal–placental unit during embryonic development. This may explain how altered nutritional status during neonatal and fetal development can affect the long-term programming of adiposity.

Leptin has also been shown to suppress apoptosis and act as a mitogen. Leptin stimulates secretion of ST and restores pulsatile ST secretion after fasting. Leptin decreases GHRH receptors but increases GHS-R in isolated pituitary somatotrophs. Thus leptin decreases GHRH stimulation while increasing GHS stimulation of ST secretion.

Leptin has direct effects on a variety of tissues and this has been shown in isolated cells and tissues treated with physiological levels of leptin *in vitro*. It reduces lipid synthesis and increases lipolysis in isolated adipocytes and fat pads. Leptin reduces the effect of insulin on stimulating gluconeogenesis in HepG2 liver cells and reduces insulin secretion by pancreatic islets. Glucose uptake and glycogen synthesis are stimulated in myotubes by leptin. Leptin acts on muscle and liver to stimulate the phosphorylation and activity of adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is a critical metabolic energy sensor that is activated by elevated AMP/ATP ratios. AMPK phosphorylates enzymes involved in fatty acid synthesis and oxidation, and transport into mitochondria, to limit the accumulation of triglycerides in these tissues. In the hypothalamus, leptin inhibits AMPK to reduce food intake and body weight. For more information, see the review by Obradovic *et al.* (2021).

Applications

Leptin can affect nutrient intake and carcass composition, including the level and rate of fattening. This, together with the effects on reproductive efficiency, makes leptin of interest to animal producers. Leptin and its receptors are potential candidate genes for the development of genetic markers for improvements in animals. In beef cattle, a variety of mutations in the leptin gene have been shown to increase feed intake and level of fatness. A commercial test (Ingenity-LR), marketed by Merial, tests for one of the most common mutations of the leptin gene in cattle. Genetic selection for desirable genotypes or sorting animals based on

their genotype may allow the use of specific feeding regimes to obtain a consistent end point of finishing. Increased feed intake would also be beneficial for transition dairy cows, when they often do not consume enough feed to support their high milk production (see Section 4.1).

Since leptin decreases the efficiency of energy utilization by increasing thermogenesis, the use of leptin antagonists may improve feed utilization and increase the efficiency of animal production. Leptin has broad effects on reproductive processes and may be useful to induce early puberty in young, thinner animals and to reduce the interval from parturition to oestrus. For more information, see Wylie (2011).

Adiponectin

Adiponectin is a 247 amino acid protein produced by mature adipocytes that increases feed intake, in contrast with leptin. It is the product of the ADIPOQ gene, which is one of the most highly expressed genes in white adipose tissue and adiponectin is present in high (μg ml^{-1}) levels in human plasma. Adiponectin increases fatty acid oxidation and glucose metabolism in muscle, reduces glucose output and enhances insulin sensitivity in liver and reduces lipogenesis in adipocytes. Adiponectin acts as a vasodilator by increasing the production of nitric oxide in the vascular endothelium. It has anti-inflammatory actions by decreasing the production of TNFα by macrophages and vascular adhesion molecules and increasing the production of IL-10, an anti-inflammatory cytokine. Deficiencies in adiponectin result in insulin resistance, glucose intolerance, hyperlipidaemia and cardiovascular disease.

Adiponectin has an amino-terminal signal sequence, a variable region containing cysteine residues that are involved in multimer formation, a collagen-like domain, and a carboxylterminal globular domain. It undergoes extensive post-translational modifications of hydroxylation and glycosylation and is secreted from adipocytes as homotrimers. These can form hexamers and high-molecular-weight (HMW) multimers, through intermolecular disulfide bonds between the conserved cysteine residues; these have a longer half-life in the circulation. There is also a globular form of adiponectin in the circulation, which is produced by cleavage of the C-terminal end of a trimer of adiponectin. The specific roles of the different forms of adiponectin have not yet been firmly established. Females have higher levels of HMW adiponectin than males, due to suppression of adiponectin synthesis by testosterone. Production of adiponectin, especially the HMW form, is decreased with increases in adipose tissue and is increased in response to fasting. It has been suggested that increased production of pro-inflammatory cytokines such as TNFα and IL-6 within the fatty tissue may inhibit adiponectin synthesis. More of these inflammatory cytokines are produced by visceral fat than by peripheral fat cells.

Two specific adiponectin receptors (AdipoR1 and AdipoR2), each with seven transmembrane domains, have been cloned, with differences in tissue-specific expression and binding affinity for the different forms of adiponectin. AdipoR1 is expressed mainly in skeletal muscle and binds the globular and trimeric forms of adiponectin. AdipoR2 is expressed mainly in liver and binds the hexameric and HMW forms of adiponectin. The action of adiponectin occurs through binding of its receptors to APPL1 (adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper-containing protein 1), an adaptor protein that acts as a facilitator of adiponectin signalling. The two receptors have different roles in glucose and lipid metabolism. AdipoR1 activates AMPK, which increases translocation of the glucose transporter 4 (GLUT4) to the cell surface and increases glycolysis by phosphorylation of phosphofructokinase. Fatty acid oxidation in liver and muscle is increased by inhibition of acetyl CoA carboxylase. AdipoR2 activates the peroxisome proliferator-activated receptor (PPARα) pathway, which increases oxidation of fatty acids and decreases the production of inflammatory cytokines. The decreased gluconeogenesis in the liver and increased glucose uptake by muscle results in an overall increase in insulin sensitivity, lowering blood glucose and tissue triglycerides. However, high levels of insulin down-regulate the expression of adiponectin receptors.

During fasting, serum levels of adiponectin increase and expression of AdipoR1 in the arcuate nucleus increases. This increases AMPK activity in the hypothalamus to promote feed intake. While leptin has been described as an 'adipostat' to limit the accumulation of adipose tissue, adiponectin has been suggested to be a starvation signal that inhibits its energy expenditure, promotes feed intake and stimulates free fatty acid utilization in peripheral tissues. For more information, see Polito *et al.* (2020).

Resistin

Resistin, also known as adipose tissue-specific secretory factor (ADSF) and found in inflammatory zone 3 (FIZZ3), was first discovered in mice (Steppan *et al*., 2001). It was named for its capacity to interfere with the action of insulin and was proposed as an adipokine that was the link between obesity and insulin resistance in type 2 diabetes mellitus. Resistin is produced during adipocyte differentiation and antagonizes the effects of insulin, decreasing glucose uptake in adipocytes, muscle cells and other tissues. In mice, resistin inhibits insulin signalling pathways, such as the expression and phosphorylation of insulin-receptor substrate (IRS) and protein kinase B (PKB, also known as Akt), increases phosphorylation of the serine residue in IRS1, and increases expression of PTP1B (protein tyrosine phosphatase 1B) and SOCS3 (suppressor of cytokine signaling 3), both negative regulators of insulin signalling.

In humans, resistin is expressed in high levels in leukocytes, including monocytes, macrophages and neutrophils. Inflammatory conditions are associated with increased levels of circulating resistin, and resistin production within human adipose tissue is thought to be due to resident non-adipocyte inflammatory cells. Human resistin induces tumour necrosis factor α and interleukin (IL)-1β, IL-6, IL-8, and IL-12 that stimulate a pro-inflammatory response. Resistin also induces the secretion of monocyte chemotactic protein-1 (MCP-1) and activation of nuclear factor-κB (NF-κB). Resistin also functions as a host defence peptide of innate immunity.

Resistin is encoded by the RETN gene. The human and mouse genes have very different promoter regions, suggesting different mechanisms of regulation, tissue distribution and functions. The mouse resistin gene also has an intron at the 3′ end that has several transcription factor recognition motifs such as peroxisome proliferator-activated receptor PPARγ, activating protein-1 (AP-1) and NF-κB.

Resistin is an 11 kDa (94 amino acids) protein in mice and 12.5 kDa (108 amino acids) protein in humans. The amino acid sequences of bovine, human and pig resistin share about 80% homology but only about 60% homology with rodent resistin. Resistin is present as trimers and hexamers in the circulatory system. It is a member of the resistin-like molecules (RELMs) family of small secreted C-terminal cysteine-rich proteins with hormone-like activity that initiate inflammatory processes. These proteins have a 10–11-Cys-rich motif at the carboxyl terminus that promotes the assembly of the globular domain of the resistin monomer through the formation of five disulfide bridges. This carboxyl-terminal globular domain has been proposed as the receptor-binding site of resistin. However, the resistin receptor has not yet been conclusively identified; potential candidate receptors are the tyrosine kinase-like orphan receptor-1 (ROR-1), the insulin growth factor-1 receptor (IGF-1R), the adenylyl cyclase-associated protein 1 (CAP1), toll-like receptor 4 (TLR4) and decorin (DCN). For more information, see Tripathi *et al*. (2020) and Li *et al*. (2021).

Inflammatory cytokines

A variety of inflammatory cytokines are produced by both macrophages and adipocytes, and obesity is considered to be a cause of chronic inflammation in which macrophages infiltrate adipose tissue. The imbalance between pro- and anti-inflammatory adipokines can result in insulin resistance and the development of metabolic syndrome, type 2 diabetes and cardiovascular disease. These adipokines include TNFα, interferon γ, interleukins such as IL-1, IL-6, IL-8 and IL-10, monocyte chemotactic protein and complement proteins. Inflammation is associated with insulin resistance and increased free fatty acids in the circulation, which can activate the innate immune response.

Pro-inflammatory cytokines are generally lipolytic and anti-lipogenic in adipocytes. TNFα inhibits differentiation of adipocytes, which reduces the lipid storage capacity, promotes lipid mobilization and impairs brown adipose tissue function. TNFα and IL-6 antagonize the anabolic effects of insulin, including the increased protein synthesis in muscle and lipid accumulation in adipose tissue.

Irisin

Irisin is an adipomyokine that is released primarily by muscle (a myokine) and also by white adipose tissue (an adipokine) in mice and to a lesser extent in humans in response to physical exercise. It stimulates the expression of uncoupling protein 1 (UCP1) in subcutaneous adipose tissue, leading to browning of white adipose tissue via the p38

mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, to increase energy expenditure and thermogenesis. Irisin was first described in 2012 and was linked to reduced weight and substantially decreased levels of glucose and insulin, indicating an improvement in insulin resistance in mice fed a high-fat diet (Bostrom *et al.*, 2012).

Irisin is a protein with 112 amino acids and a molecular weight of 12 kDa, which is conserved in human, mouse, rat and cattle. It is produced by cleavage of the transmembrane fibronectin type III domain-containing protein 5 (FNDC5) to release its fibronectin domain, which is then glycosylated and dimerized to form irisin. FNDC5 expression is stimulated by the transcription co-factor peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) that is involved in many pathways related to energy metabolism. Physical exertion induces expression of PGC1, leading ultimately to increased levels of irisin.

There has been considerable controversy in this area, with questions about the specificity of antibodies used to detect irisin in Western blots and the validity of ELISA assays used to quantify irisin levels in tissues. The concerns about the quality and accuracy of results from clinical studies are similar to those raised in the past about several other hormones such as LH, ST and leptin, so more reliable assays for this area need to be further developed. For more information, see Perakakis *et al.* (2017) and Maak *et al.* (2021).

3.6 Thyroid Hormones

Key concepts

- Thyroid hormones regulate metabolic rate to increase heat production and oxygen utilization.
- Thyroid hormones also regulate cell differentiation and growth and are necessary for proper brain development and for metamorphosis in amphibians.
- The synthesis of the prohormone thyroxine (T_4) in the follicles of the thyroid gland is stimulated by thyroid-stimulating hormone (TSH). T_4 is metabolized and converted to active T_3 by deiodinases in the target tissues to control the amount of active hormone in different tissues.
- Thyroid hormones act via nuclear receptors to regulate gene expression or by binding to cytosolic proteins and activating intracellular cascades.
- There is an interaction between growth hormone and thyroid hormones in regulating growth.

Thyroid hormones regulate two main types of processes. First, they affect metabolic pathways to modulate oxygen consumption, basal metabolic rate and lipid, carbohydrate and protein metabolism. There are also secondary effects due to altering the levels of other hormones. Secondly, thyroid hormones trigger cell differentiation and maturation in a number of tissues and indirectly affect growth. These effects on metabolism and cell differentiation are interrelated.

Synthesis and metabolism

The metabolism of thyroid hormones is illustrated in Fig. 3.20. Thyroid hormone is synthesized in the follicles of the thyroid gland and this is stimulated by TSH released from the anterior pituitary (see Section 1.2 and Fig. 1.17). The precursor of thyroid hormones is a large glycoprotein called thyroglobulin, which contains tyrosine residues that are iodinated by thyroperoxidase (TPO). These residues are coupled to form iodinated thyronine, mainly in the form of the prohormone tetraiodothyronine or thyroxine (T_4). Hydrolysis of thyroglobulin causes the release of thyroid hormones, mostly as T_4. Thyroid hormones are highly hydrophobic and are bound to transthyretin as well as thyroid hormone-binding globulin for transport and stabilization in the blood. Thyroid-binding globulin is not found in birds.

The metabolism of thyroid hormones is carried out by type 1, 2 and 3 deiodinases (DIO1, DIO2 and DIO3). These enzymes are expressed in a tissue-specific manner and act to regulate the activity of thyroid hormones in different tissues (Fig. 3.21). DIO2 catalyses 5′-deiodination and converts T_4 to the most biologically active form of thyroid hormone, triiodothyronine (T_3). DIO2 also converts reverse T_3 (rT_3) to diiodothyronine (T_2). T_4 and T_3 are metabolized to the less active rT_3 and T_2 by 5-deiodination catalysed by DIO3. The DIO1 enzyme catalyses both 5- and 5′-deiodination and can act on a variety of different iodothyronines.

The deiodinase enzymes are selenium-dependent enzymes with active selenocysteine residues. A potent inhibitor of the DIO1 enzyme is 6-propyl-2-thiouracil (PTU), while all of the deiodinase

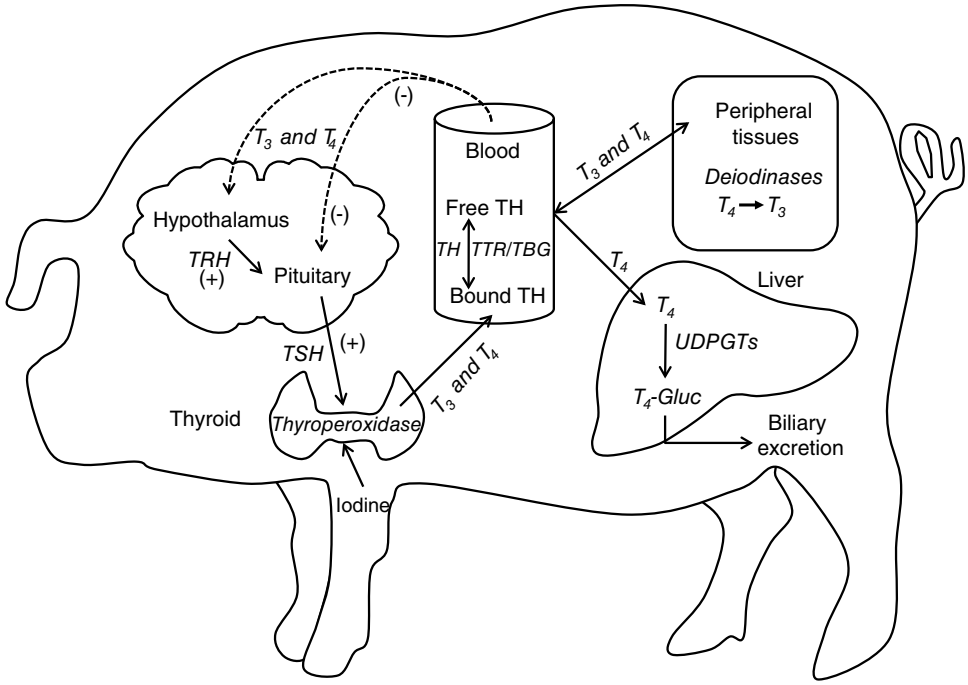


Fig. 3.20. Summary of thyroid hormone metabolism. TH, thyroid hormone; TTR, transthyretin; TBG, thyroid hormone-binding globulin.

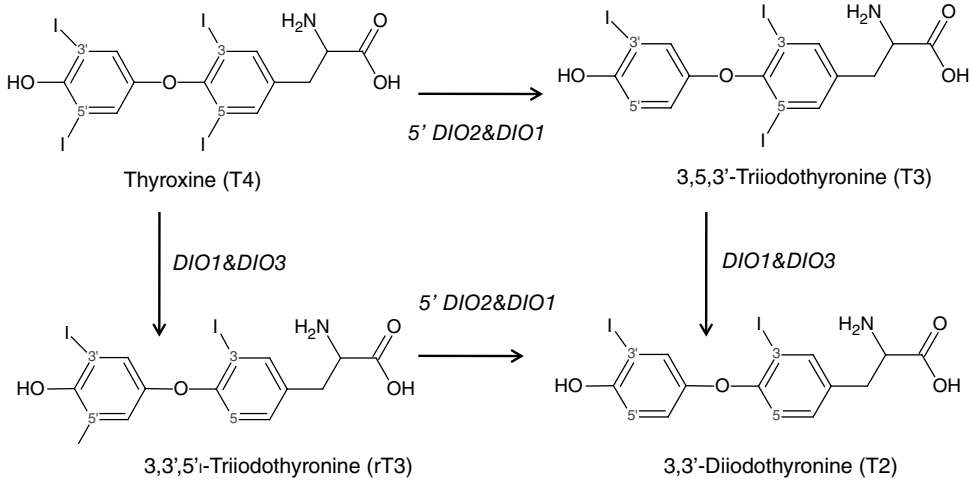


Fig. 3.21. Metabolism of thyroid hormones by deiodinases.

enzymes are inhibited by iopanoate. The tissue-specific expression patterns of DIO2 and DIO3 suggest that they play an important role in the local and systemic availability of active thyroid hormone. Thus, DIO2 increases levels of T_3, while DIO3 decreases the amount of active T_3; together these enzymes regulate T_3 levels within very narrow limits in specific tissues to avoid either an excess or deficiency of T_3. The DIO1 enzyme is thought to act mainly as a scavenger enzyme to reclaim iodine

before the inactivated thyroid hormone is excreted. Thyroxine can also be deaminated to form inactive tetraiodothyroacetate, or conjugated to glucuronides by UDP-glucuronyl transferase or sulfoconjugated by phenol sulfotransferase and then excreted. For more information on deiodinase enzymes, see the review by Luongo *et al*. (2019).

For interest

A common polymorphism of DIO2 (Thr92Ala) affects the stability and activity of the enzyme, which reduces conversion of T_4 to T_3. This could reduce the amount of active thyroid hormone and may be associated with some metabolic diseases and mental disorders.

Another thyroid hormone metabolite, 3-iodothyronamine (T1AM), has been identified which exerts actions partly opposite to and distinct from known functions of thyroid hormones. T1AM dramatically decreases body temperature and heart rate, and shifts fuel utilization from carbohydrates to lipids. T1AM is presumably produced by decarboxylation and deiodination of thyroid hormones but the details of the biosynthetic pathway have not yet been established. Analytical methods for measuring T1AM in biological samples are also in need of further development. It circulates largely bound to lipoprotein particles, particularly to apolipoprotein B100 (apoB100).

T1AM is not a ligand of nuclear thyroid hormone receptors but acts at the cell surface via the G protein-coupled receptors, trace amine associated receptor 1 (TAAR1) and the α2A-adrenergic receptor (ADRA2A), and intracellularly via mitochondrial F0F1-ATP synthase. This explains the rapid metabolic and hypothermic effects of T1AM which occur too rapidly to directly involve gene transcription. However, T1AM also modulates gene expression in its target tissues, especially liver, adipose tissue and skeletal muscle. It up-regulates several genes in adipose tissue and liver involved in lipoprotein functions and has regulatory effects on genes involved in lipolysis and β-oxidation. In skeletal muscle, T1AM stimulates expression of genes involved in fatty acid oxidation and turnover. For more information, see the reviews by Köhrle and Biebermann (2019) and Rutigliano *et al*. (2020).

Transporters and receptors

Thyroid hormone release and synthesis are stimulated by TSH, which is synthesized in the basophilic thyrotrophs of the pars distalis in the anterior pituitary. TSH is released from the pituitary in response to TRH from the hypothalamus, which is a tripeptide (pyro-Glu-His-Pro-NH_2) that has the same structure in all vertebrates. The amino acid sequence of TSH is highly conserved and TSH from mammalian sources stimulates thyroid activity in most vertebrates. TSH interacts with receptors in the thyroid follicular cells to activate adenylate cyclase and increase cAMP production, resulting in increased synthesis and release of thyroid hormones. Thyroid hormones exert a negative feedback on TSH secretion as well as the hypothalamic TRH. This feedback is dependent on the presence of functional thyroid hormone receptors, which bind T_3 and repress the expression of TSH and TRH genes.

Thyroid hormones act via two different mechanisms. There is a direct action of T_4, rT_3 or T_2 on subcellular organelles or the plasma membrane by binding to receptors such as integrin αvβ3. T_3 also acts by binding to nuclear receptors and affecting gene expression. Thyroid hormones are lipophilic and it was originally thought that they diffuse across the cell membrane; however, it is now known that they are transported across the cell membrane mainly via the monocarboxylate transporter 8 (MCT8 encoded by the *SLC16A2* gene). Variations in the activity of this receptor may provide another level of regulation of thyroid hormone activity within a particular tissue (Grijota-Martínez *et al*., 2020)

For interest

The *SLC16A2* gene is on the X chromosome and mutations in the gene that alter the function of the MCT8 transporter protein will limit the levels of T_3 during brain development. This may be the molecular basis behind some X-linked forms of neurological problems (Allan–Herndon–Dudley syndrome) that result in limb spasticity and mental illness.

There are three major thyroid hormone receptors (TRα1, TRβ1 and TRβ2), which are encoded by two different genes. TRα1 and TRβ1 are expressed in almost all tissues, but TRα1 is particularly

abundant in brain, heart and the immune system, while TRβ1 is more abundant in brain, liver and kidney. The expression of TRβ1 is developmentally regulated. The TRβ2 receptor is expressed in the hypothalamus and pituitary and is responsible for the negative feedback of T_3 on TSH and TRH synthesis. In the absence of T_3, the receptors bind repressors NCoR (nuclear receptor co-repressor) and *SMRT* (silencing mediator of retinoic acid receptors and thyroid hormone receptors) and histone deacetylases, which repress gene expression below basal levels. Conversely, when T_3 is bound, the receptors release the co-repressors and bind co-activators (p160 SRC) that have histone acetylase activity and activate gene transcription.

Metabolic effects

The effects of thyroid hormones on metabolism and growth and development are summarized in Fig. 3.22. Thyroid hormones increase heat production and oxygen utilization by heart, liver, kidney and pancreas. In chickens, a precocial species, there is an increase in thyroid activity in the peri-hatch period in response to cooling, while doves, an altricial species, show little thermoregulatory development until 1–2 weeks after hatching. At older ages in chickens, the conversion of T_4 to T_3 is increased by short-term cold exposure and decreased by warm temperatures. Norepinephrine also plays an important regulatory role in non-shivering thermogenesis and thyroid hormones play a permissive role for this action of catecholamines by increasing the number of β-adrenergic receptors.

The maximal rate of stimulation of the basal metabolic rate (BMR) by thyroid hormone, as measured by oxygen consumption, is 100–150% in humans. Heat production involves stimulation of the Na^+/K^+ ATPase ion pump. The thermogenic effects of thyroid hormones are mediated through both short-term (minutes) and long-term (hours) effects on mitochondria. The short-term effects are due to T_3 binding to TRα1 on the inner mitochondrial membrane. Inhibitors of protein synthesis do not affect these short-term effects, which can also be demonstrated in isolated mitochondria, so they do not involve gene transcription. The long-term effects occur at the nuclear level, which include changes in phospholipid turnover and increased synthesis of uncoupling protein (UCP), which increase proton leakage at the inner membrane. T_3 binds to TRα1 in the inner mitochondrial matrix to stimulate mitochondrial genome transcription. T_3 is also involved in the production of new mitochondria, by activating both nuclear and mitochondrial genome expression.

In the liver, T_3 stimulates the synthesis of malic enzyme, which converts malate to pyruvate and provides NADPH for lipogenesis. Thyroid hormones also increase glucose absorption from the gastrointestinal tract, and its uptake and oxidation, at the same time dampening glucose-stimulated insulin release and increasing gluconeogenesis. Insulin increases the effects of T_3 but has little effect by itself, while glucagon is inhibitory. The effects are not seen on malic enzyme in non-hepatic tissues, indicating that tissue-specific factors are important. In chickens, malic enzyme does not

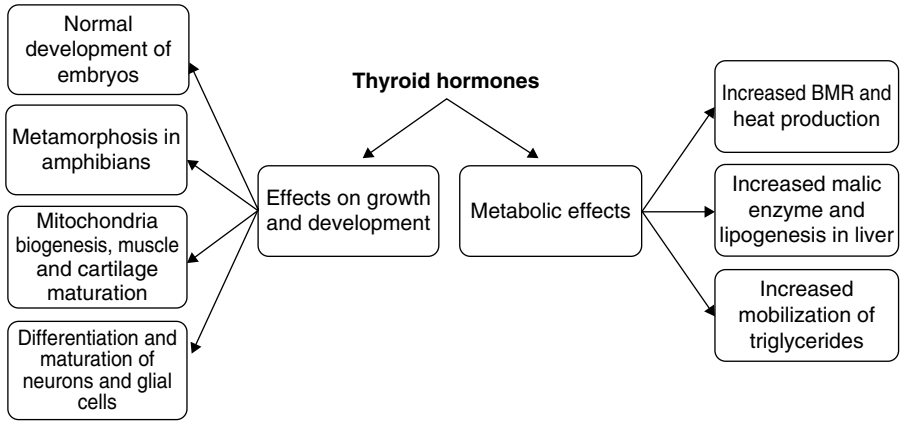


Fig. 3.22. Effects of thyroid hormones on metabolism, growth and development.

increase in response to T_3 during the peri-hatch period but only after the birds begin to feed in the early post-hatch period, when carbohydrate from the diet becomes the primary energy source.

Thyroid hormones affect not only growth but also carcass composition. Thyroid hormones increase the activity of lipoprotein lipase to increase the mobilization of triacylglycerol from adipose tissue and induce the transcription of enzymes required for fatty acid synthesis, such as acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS), and stimulate lipolysis. Thyroid hormone also improves the clearance of cholesterol and its conversion to bile acids for bile secretion. Low levels of thyroid hormones in poultry are associated with increased fatness, while hyperthyroidism reduces abdominal fat. Similarly, pigs exposed to high temperatures have decreased levels of thyroid hormones and increased uptake and storage of triacylglycerols in adipose tissue.

Levels of T_3 decrease during fasting and increase again during re-feeding. This is due to changes in the conversion of T_4 to T_3, as well as changes in TSH release from the pituitary. The levels of dietary carbohydrate affect both of these factors. It is likely that increases in heat production and basal metabolic rate from increased levels of T_3 lower feed efficiency and growth.

Effects on growth and development

Thyroid hormones are necessary for proper growth and development and a deficiency in thyroid hormones results in severe growth retardation. Thyroid hormones coordinate embryonic and early postnatal development by regulating the expression of key developmental genes that are responsive to thyroid hormone signalling during specific time windows. Hypothyroidism during early stages of development results in deficiencies in somatic, neural and sexual development and decreased metabolic rate. Early embryos lack a functional thyroid gland, so while mammalian embryos/fetuses can receive maternal thyroid hormone via the placenta, developing avian embryos rely on thyroid hormones that are deposited in the egg yolk. Inadequate thyroid hormone production leads to high levels of TSH and hypertrophy of the thyroid gland to cause goitre. This was traditionally a problem in humans when dietary iodine was inadequate. Thyroid deficiency also results in decreased wool growth in sheep and hair growth in a number of different species. Feather development is also affected by thyroid hormones. It has been known for a long time that thyroid hormones are necessary for metamorphosis in tadpoles and this is the basis for a bioassay for endocrine disruptor chemicals that affect thyroid hormones (see Section 6.4). In the rat, plasma T_3 and T_4 increase during the first 3 weeks after birth and then decrease to adult levels. For more information on thyroid hormone in birds, see Darras (2019).

There is an interaction between ST and thyroid hormones. Thyroid hormones act in a permissive or indirect manner and are required for secretion of ST and for its systemic actions. Both thyroxine and ST increase the production of muscle proteins, and there is a synergistic action of both hormones on muscle and whole-body growth. In birds, conversion of T_4 to T_3 is increased by pulsatile administration of ST but not by continuous ST administration. Sex-linked dwarf chickens, which are deficient in 5′-deiodinase activity, are an important model for studying the effects of thyroid hormones on growth.

Mitochondria have been implicated in the control of cellular proliferation and apoptosis. T_3 affects mitochondrial biogenesis and activity and thus is involved in the differentiation and maturation of various cell types. These include neurons and glial cells of the central nervous system. Levels of T_3 are maintained in the brain during periods of hypothyroidism by increases in the activities of the DIO2 deiodinase, which converts T_4 to T_3, and decreases in the activity of the DIO3 deiodinase, which converts T_3 to diiodotyrosine. T_3 is also a major regulator of myoblast differentiation in muscle development and in chondrocyte hypertrophy in cartilage maturation. There is decreased T_3 in the early phase of satellite cell activation which enables the proliferation and expansion of satellite cells. T_3 activity is increased in the later stages of myocyte differentiation, which results in enhanced differentiation and myotube formation for muscle growth.

Thyroid hormone is also involved in seasonal effects on reproduction. Seasonal changes in production of melatonin by the pineal gland regulates the expression of DIO3 and DIO2. The activity of these deiodinases controls the levels of T_3, which activates genes encoding the neuropeptides RFRP3 (RF-amide-related peptide-3, GnIH) and kisspeptin (KISS1), which regulate the secretion of GnRH (see Section 5.1)

Applications

Thyroprotein (iodinated casein) stimulates milk production in dairy cows when given at certain stages of lactation. Adding TRH to the diet of lactating sows has been shown to increase plasma T_4, ST and prolactin and increase milk production and weaning weights of piglets. However, sows fed thyroprotein took longer to return to oestrus after weaning and had smaller litters in subsequent lactations. Adding thyroprotein to the diet can also increase growth rates and feed efficiency of grower/finisher pigs but this results in a higher percentage of fat and lower percentage of protein in the carcass.

Supplementation of broiler chicken diets with T_3 at 0.1 mg kg^{-1} diet stimulated growth, while higher levels (0.3 mg kg^{-1} diet) depressed growth. Levels of T_3 can also be affected by altering the selenium status of broilers, since this affects the activity of the deiodinase enzymes. Growth is impaired in selenium-deficient broilers since they cannot convert T_4 to T_3.

Feather development is under the control of thyroxine. T_4 levels are high and T_3 levels are low during the initiation of the moult, but T_4 levels decline and T_3 levels gradually increase as new feathers start to grow. Thyroid hormones or severe feed restriction can induce moulting. High levels of thyroid hormone during moulting may also be required for thermoregulation during a period of heavy feather loss. Thyroid hormones are required for reproductive system development, but high levels of thyroid hormones during moulting have anti-gonadal effects (see Section 6.1).

Ascites in broiler chickens is caused by an imbalance in the amount of oxygen required for tissue growth and development and the amount of oxygen that is available to the tissues (see Section 3.9). Increased thyroid hormone levels increase oxygen utilization and this can lead to increased incidence of ascites.

3.7 β-Adrenergic Agonists

Key concepts

- β-Adrenergic agonists (β-AAs) are orally active synthetic analogues of epinephrine and norepinephrine that act via β-AA receptors.
- β-AAs stimulate lipolysis and decrease lipogenesis in adipose tissue to decrease carcass fat, and stimulate protein accretion and hypertrophy of skeletal muscle.
- β-AAs repartition nutrients to increase carcass lean yield and improve feed efficiency but they may cause some negative effects on meat quality.
- The response to β-AAs decreases over time, so a relatively short treatment time towards the end of the finishing period is best.

β-Adrenergic agonists (β-AAs) are synthetic, orally active compounds that are mostly phenethanolamine derivatives related to the naturally occurring catecholamines, dopamine, noradrenaline (norepinephrine) and adrenaline (epinephrine). Norepinephrine functions both as a neurotransmitter in the sympathetic nervous system and as an endocrine hormone produced by the adrenal medulla. It is biosynthesized from tyrosine in the adrenal medulla (see Fig. 1.19) and is present in plasma at two to five times the levels of epinephrine and dopamine. Epinephrine is synthesized by methylation of norepinephrine (see Sections 1.2 and 6.3).

Catecholamines regulate a variety of physiological functions, including the speed and force of heart contractions, motility and secretions from various parts of the gastrointestinal tract, bronchodilation, salivary gland and pancreas secretions, brown adipose tissue metabolic activity, blood vessel dilation and contraction, uterine contraction and spleen capsule contraction. This wide range of processes is regulated by the presence of distinct groups of α- and β-receptors in different tissues. Epinephrine is more potent than norepinephrine for α-receptors. Norepinephrine is more potent than epinephrine for β1-receptors.

Mechanism of action

In stressful situations, the sympathetic nervous system stimulates the release of the catecholamines, epinephrine and norepinephrine, from the adrenal medulla (see Sections 3.12 and 6.3). These hormones act on α- and β-receptors that are located in many different tissues to produce the 'fight or flight' response. Activation of the β-receptors increases the availability of metabolic fuels (glucose and fatty acids) and the dilation of airways increases the availability of oxygen. Catecholamines stimulate the activity of glycogen phosphorylase and inhibit glycogen synthase to increase the conversion of glycogen to glucose in muscle. These effects are also stimulated by the β-AA analogue isoproterenol and

are inhibited by the β-receptor antagonist propanolol. Catecholamines stimulate lipolysis and inhibit lipogenesis in adipose cells and these effects can be blocked with β-adrenergic receptor antagonists.

β-AAs are used in both human and veterinary medicine and have been used in the treatment of asthma since the 1970s because of their ability to relax smooth muscle in the airways. They are given in small doses directly into the airways using an inhaler, so that little of the drug enters the circulation to affect other tissues. β-AAs are also effective as repartitioning agents that alter nutrient metabolism to produce dramatic increases in lean and decreases in adipose tissue growth in livestock.

β-AA structures

Several synthetic analogues of epinephrine and norepinephrine have been investigated for their effects on increasing skeletal muscle growth and decreasing the fat content of carcasses. The phenethanolamine β-AAs have the same overall structure (phenyl-CH(OH)-CH_2-NH-R), but individual compounds may have quite different chemical and pharmacokinetic properties. In order to be biologically active, these β-AAs must have a substituted six-membered aromatic ring, a hydroxyl group on the β carbon and a positively charged nitrogen on the ethylamine side chain. A bulky R group on the side chain confers specificity for the β-receptor. The substituents on the aromatic ring at the 3 and 4 carbons are important, since they can form hydrogen bonds to a serine hydroxyl in the β-receptor. Substituting the hydroxyl groups on the aromatic ring for halogen atoms (e.g. clenbuterol) prevents the rapid catabolism and deactivation of β-AA by oxidative and conjugative pathways. In contrast, β-AAs having hydroxylated aromatic rings are readily metabolized, typically by conjugation, and have relatively short plasma half-lives (e.g. ractopamine).

Common phenethanolamine β-AAs include cimaterol, clenbuterol, ractopamine and L644,969; zilpaterol and lubabegron are examples of β-AAs that are not a phenethanolamine (Fig. 3.23). Each of these compounds can have multiple actions on

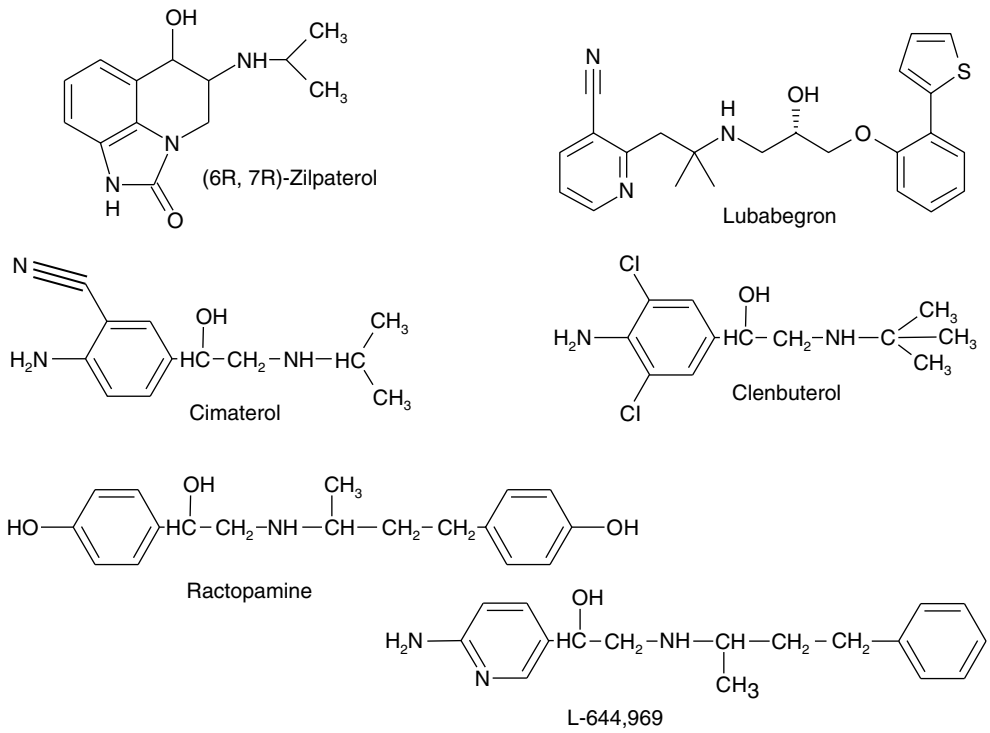


Fig. 3.23. Structures of β-adrenergic agonists.

various aspects of nutrient metabolism, which can vary in different species. The positive response to protein accretion is largely confined to skeletal muscle.

β-AA receptors

Most tissues have a mixture of α- or β-adrenergic receptors, with different amounts of each receptor type present in different tissues and in different species. Cloning of β-AA receptors has confirmed the existence of α1-, α2- and β1-, β2- and β3-receptors. β-AA receptors within a species have about 50% amino acid sequence homology, while individual β-AA receptor subtypes have 75% sequence homology across species. The β-AA receptors are glycosylated with an approximate molecular mass of 65 kDa.

β-AAs act by binding primarily to β2-adrenergic receptors but most β-AA compounds used in animal agriculture bind to more than one type of β-receptor. Ractopamine and zilpaterol bind to both β1- and β2-receptors found throughout the body, including in the muscle and adipose tissue. Another group of compounds, selective β-adrenergic receptor modulators, have agonistic effects on specific receptor subtypes and antagonistic effects on other receptor subtypes. Lubabegron is a newly developed selective β-adrenergic receptor modulator that is antagonistic for β1- and β2-receptors but agonistic for the β3-receptor. Lubabegron was approved in 2018 for use in cattle to reduce ammonia emissions during the last 14–91 days prior to slaughter. This is likely due to increased efficiency of tissue accretion, thereby decreasing nitrogen excretion.

Some tissues have predominantly one particular receptor subtype. Stimulation of β1-receptors in heart muscle can result in tachycardia, while guinea pig tracheal muscle has primarily β2-receptors. These tissues were used to classify the specificity of different β- agonists and antagonists for different receptor types. For example, the compound CGP 20712A is a specific β1-receptor antagonist; ICI 118,551 is a specific β2-receptor antagonist; and SR 59,230A is a specific β3-receptor antagonist. Isoproterenol is a universal β-receptor agonist, while propranolol is a β1/β2-receptor antagonist. Compounds with a high degree of specificity for a particular receptor subtype were then used to classify different tissues for the receptor subtypes present. However, there are species differences in the effectiveness of different β-AAs for subtypes of β-receptors. There may also be differences in the cellular distribution of the different β-receptor subtypes.

Heart contractility is stimulated primarily by β1-receptors, although β2-receptors are also present in heart muscle in some species. β2-Receptors are also found in blood vessels and aid in vascular dilation. In cattle and sheep, skeletal muscle and adipose tissue have β2-receptors. The β3-receptor subtype is the predominant receptor in brown and white adipose tissue in rats and in brown adipose tissue in the bovine fetus. It is pharmacologically different from β1 and β2 and is not as readily inactivated by phosphorylation or internalization.

Binding of β-AA to its receptor activates the $G_{\alpha s}$ protein, to activate adenylate cyclase and increase cAMP to activate protein kinase A, which phosphorylates a number of intracellular proteins (see Section 1.3). Hormone-sensitive lipase, the rate-limiting enzyme responsible for triacylglycerol degradation in adipocytes, is activated by phosphorylation. Acetyl-CoA carboxylase, which is the rate-limiting enzyme for fatty acid biosynthesis, is inhibited by phosphorylation (Fig. 3.24). The number of insulin receptors on adipocytes is also reduced. Together, this results in decreased lipogenesis and increased mobilization of fat from adipose tissue. In muscle, β-AAs stimulate the activity of glycogen phosphorylase, thereby enhancing the conversion of glycogen to glucose, and inhibit glycogen synthase, reducing the conversion of glucose to glycogen. β-AAs also enhance the uptake of gluconeogenic and branched-chain amino acids needed for protein synthesis in muscle. In addition, they are responsible for reducing the activity of calpains, which decreases muscle protein degradation.

The CREB protein is also phosphorylated by protein kinase A and subsequently binds to the cAMP response element in the regulatory region of genes to stimulate transcription of muscle-specific proteins, such as α-actin and myosin light chains. Increased levels of cAMP in blood vessels inhibit myosin light-chain kinase, which is responsible for the phosphorylation of smooth muscle myosin, resulting in vasodilation and improved blood flow. Thus, β-AAs increase muscle protein deposition and lipid catabolism to improve carcass lean yield.

The β-AA receptor is eventually inactivated by uptake and metabolism of the β-AA. Norepinephrine and epinephrine are catabolized by catechol-*o*-methyl transferase, which is an enzyme that methylates the hydroxyl groups on the aromatic ring,

and by deamination by monoamine oxidase. The β-AA receptor can also be inactivated by phosphorylation of the receptor after β-AA binding by protein kinase A (see Section 1.3). Chronic stimulation with β-AA also reduces the number of β-AA receptors.

Physiological responses to β-AA

The effects of β-AAs are generally greatest in cattle and sheep and least in chickens, with pigs being intermediate, but the results are quite variable (Table 3.4). This may be due to the fact that some species, such as broiler chickens, have been intensively selected for growth and are closer to their biological maximum growth rate. There may also be differences in the ability of different β-AAs to activate receptors in different species.

β-AAs increase muscle growth in both obese and lean genotypes of pigs but the genotype differences are not eliminated by β-AA treatment. The effects of β-AAs on growth performance and carcass composition are much smaller in young pigs and ruminants that are not accumulating fat at a fast rate compared with market-weight animals. It is not known whether this is due to low receptor numbers or increased desensitization of receptors in younger animals.

Carcass fat is reduced by 20–30% in cattle and sheep, with less dramatic improvements of about 10% in pigs. This may be due to rapid down-regulation of β-AA receptors in pig adipocytes and their relative insensitivity to β-AA. Small but significant improvements in dressing percentage and feed conversion are found. β-AAs are also effective in poultry but the effects are less than those seen in mammals. Cimaterol reduces carcass fat by 10% in female broilers and 5% in male broilers. Dose-dependent improvements in growth and carcass composition are also found in ducks and turkeys.

The effects of β-AA on metabolism are summarized in Fig. 3.25. Insulin regulates the rate of lipogenesis, and β-AAs reduce the rate of lipogenesis through a reduction in the number of insulin receptors and in the binding of insulin to adipocytes. β-AAs also act directly on adipose tissue via the β-receptor to stimulate lipolysis and decrease lipogenesis.

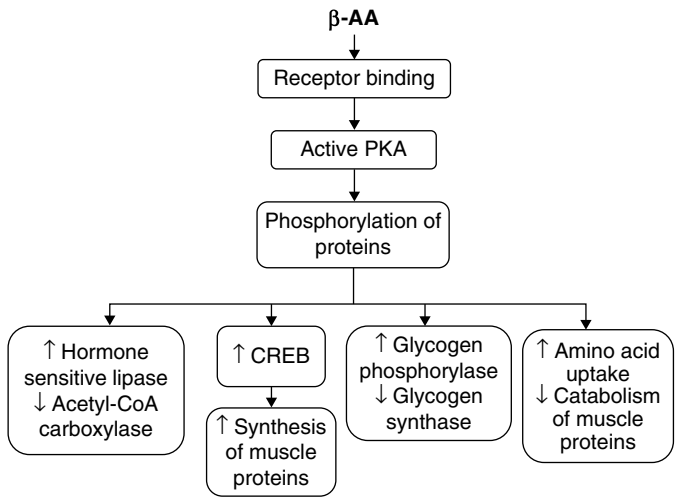


Fig. 3.24. Mechanism of action of β-adrenergic agonists.

Table 3.4. Effects of oral β-AA in different species (% change) (from Mersmann, 1998).

Animal	Weight gain	Feed consumption	Gain/feed	Muscle	Fat
Cattle	+10	−5	+15	+10	−30
Sheep	+15	+2	+15	+25	−25
Pigs	+4	−5	−5	+4	−8
Chicken	+2		+2	+2	−7

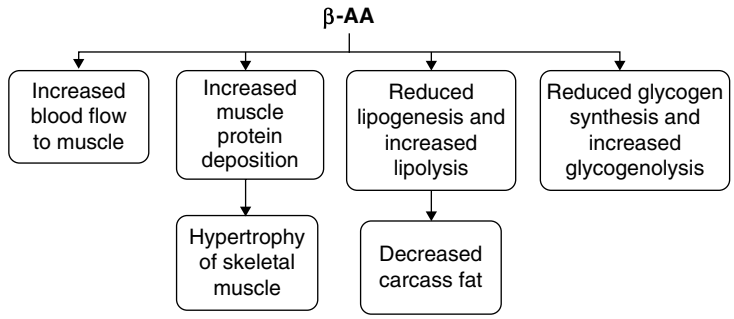


Fig. 3.25. Metabolic effects of β-adrenergic agonists.

Increased lipolysis by β-AA results in acutely increased levels of non-esterified fatty acids in the plasma of pigs and cattle.

β-AA treatment causes muscle hypertrophy rather than hyperplasia. The response is greater in type II fibres (fast-contracting, mixed glycolytic–oxidative) than in type I fibres (slow-contracting, oxidative). Long-term treatment with β-AAs may increase the proportion of type II fibres in a muscle.

β-AA treatment can also increase blood flow to the skeletal muscle and thereby increase the availability of energy and amino acids required for protein synthesis. Increased blood flow to the adipose tissue may help to remove the non-esterified fatty acids produced from lipolysis.

The effects of β-AAs on muscle metabolism are probably mediated by direct effects rather than indirect effects on other endocrine systems, since the effects of β-AA are also seen in hypophysectomized or severely diabetic rats. Thus, the mode of action of β-AA is by direct receptor-mediated stimulation of muscle growth. Treatment with β-AA can both increase protein synthesis and decrease the degradation of muscle protein. Increased levels of mRNAs for muscle-specific proteins, such as myosin light chain and α-actin, as well as reduced activity of calpains and other specific proteolytic systems, have been reported.

Dry matter intake is commonly reduced after initial exposure to β-AA but this returns to normal after a short period. The repartitioning effects of β-AA occur in both feed-restricted and adequately fed animals but the increase in growth rate is only seen in well-fed animals.

The increased protein deposition with β-AA is accompanied by a proportional increase in dietary protein requirements, and no response to ractopamine was found in pigs when dietary crude protein levels were less than 140 g kg^{-1}. At higher dietary protein levels, the maximal protein deposition rate is 23% higher in ractopamine-treated gilts compared with controls. This suggests that β-AAs increase the maximum rate of protein deposition. Sufficient levels of dietary energy are required to sustain the increased rate of protein deposition with β-AA treatment. However, maintenance energy requirements are not increased by β-AA. This may be because the energy requirements of increased muscle mass are offset by decreased energy requirements of a smaller visceral mass.

An important advantage of β-AAs is that they stimulate protein deposition primarily in skeletal muscle, while pST stimulates protein deposition and growth in all tissues. The increased energy and protein required for muscle synthesis comes both from increased utilization of dietary energy and protein and from turnover of other body tissues, such as liver, kidneys and gut. The weight of the liver, heart and gut are often reduced by β-AA treatment. There is little or no effect of β-AAs on bone weight in ruminants but β-AA treatment results in decreased bone weight in pigs. Thus the dressing percentage (ratio of carcass to live weight) is increased by β-AA treatment.

The effects of β-AAs on meat quality can vary with the different β-AAs used, the species, dose and length of treatment. Overall, the anabolic effects of β-AAs tend to increase meat toughness, as measured by shear force values. Trained sensory panels found decreased tenderness in beef, but not in pork, due to inhibition of the calpain/calpastatin system. Other objective measures of meat quality, including pH, colour, drip loss and protein solubility, are not affected. There is some risk of developing DFD meat, due to the decreased muscle glycogen and plasma glucose at slaughter (see Section 3.12).

Delivery/dose

β-AAs are absorbed readily from the gut with peak plasma concentrations occurring 1–3 h after oral administration. They are then distributed through the plasma to various tissues, such as muscle, adipose tissue, liver, kidney, brain and lungs, and are metabolized both in the intestine and in the liver.

The effects of β-AA are dose dependent, with a tendency for reduced effects at very high doses. Doses of 5 μg kg^{-1} BW resulted in peak plasma levels of 0.5 ng ml^{-1} of clenbuterol after 3–5 h in cattle, with plasma levels increasing to 2 ng ml^{-1} as the treatment is continued. Doses of 50–80 μg kg^{-1} BW of salbutamol resulted in plasma levels of about 5 ng ml^{-1}. For pasture-fed cattle, use of β-AAs as a feed additive is impractical, since they are not available as implants.

The response to β-AAs decreases over time if they are administered at a constant rate on a daily basis. The initial marked response to β-AA peaks at 14 days after treatment and then is greatly decreased by 21 days of treatment. This occurs through densensitization of receptors and uncoupling of receptors with the adenyl cyclase system and down-regulation of receptor numbers in the cell membrane. Thus, a relatively short treatment time towards the end of the finishing period will maximize the response to β-AA.

A withdrawal period can reduce the overall effectiveness of β-AA treatment. The withdrawal period should be kept relatively short (about 5 days), since compensatory growth of adipose tissue is seen after withdrawal of β-AA, while muscle mass is maintained.

Safety aspects

The oral potencies of different β-AAs can differ by as much as three orders of magnitude, due to differences in the structures, pharmacokinetics and metabolism of the different β-AAs. β-AAs that have halogenated aromatic ring systems, such as clenbuterol, are metabolized by both oxidative and conjugative pathways and have a long half-life. β-AAs that have hydroxylated aromatic rings are metabolized solely by conjugation and have a short half-life. The metabolism occurs in the liver and in the intestine during absorption.

The β-AAs are all orally active, in contrast to ST and most anabolic steroids. β-AAs are also heat stable and are not destroyed by cooking. This increases concern about the potential effects of residues of β-AA in meat. The half-life estimate for plasma cimaterol in steers was 54 min, and for plasma clenbuterol in veal calves it was 18 h for the initial distribution phase and 55 h for the terminal half-life. These estimates are much larger than for epinephrine. The very long half-life for clenbuterol means that high concentrations can accumulate in liver and adipose tissue. The effective therapeutic dose for clenbuterol is 10–20 μg with a no observed effects limit (NOEL) of 2.5 μg day^{-1}. In contrast, the effective dose for other common β-AAs is 2000–10,000 μg. These less potent β-AAs suggest that safe and effective use of β-AAs is possible.

Ractopamine hydrochloride was approved for use in finishing pigs (Paylean®, from Elanco) in the USA in 1999 and in Canada in 2005. Paylean fed at 18 g t^{-1} of feed improved feed efficiency by 13%, increased average daily gain by 10%, reduced average daily feed intake by 6% and increased lean gain by 25–37% in research trials. It is fed for 28 days before slaughter with no withdrawal time. For more information, see Aroeira *et al.* (2021).

Ractopamine (Optaflexx®) and zilpaterol hydrochloride (Zilmax®) are also approved for cattle in the USA, but the sale of Zilmax by Merck in the USA and Canada was suspended in 2013 due to problems with lameness and lost hooves. In Canada and Europe, clenbuterol is approved only for therapeutic use as a bronchodilator in horses and calves and for tocolysis to prevent premature birth in cows. Because of its high potency, clenbuterol has been used illegally in cattle and this led to the hospitalization of 135 people in Spain in 1990 after clenbuterol poisoning caused by calf liver contaminated with 160 and 291 μg of clenbuterol kg^{-1}. The symptoms include muscle tremors, cardiac palpitations, nervousness, headache, muscular pain, dizziness, nausea, vomiting, fever and chills. The highest accumulation of clenbuterol occurs in pigmented tissues such as hair and the retina of the eye, due to binding with melanin. This finding can be used for a highly sensitive test for detecting residues of clenbuterol up to 60 days after treatment. Intensive monitoring programmes have shown a decrease in the illegal use of clenbuterol in the Netherlands, Germany, Northern Ireland and Spain by the mid-1990s. In the USA, clenbuterol has been used illegally for some showring animals to accentuate muscle definition and reduce subcutaneous fat. For more information on the risk assessment of using β-AAs, see the review by Dilger *et al.* (2021).

Alternative approaches for using growth promoters

The effect of combined use of growth promoters depends on differences in the mode of action of the different compounds. An additive effect can be expected when two growth promoters act via different mechanisms. Anabolic steroids and β-AA increase meat yield by reducing protein degradation more than by increasing protein synthesis, while ST increases protein synthesis more than reducing protein degradation. Studies in pigs and veal calves combining ST treatment with β-AAs showed additive effects on feed efficiency and loin eye area from the two treatments.

Vaccines can potentially be used to mimic the effects of β-AA and ST, without the need to administer natural or synthetic hormones. This might alleviate concerns about the use of hormonal growth promoters and also make it feasible to improve growth in animals on extensive grazing systems.

Antibodies can be raised against natural hormones to block their effects. Targets for this research have been somatostatin, which blocks ST release, and ACTH, which stimulates release of the catabolic hormone cortisol. Antibodies can also be generated that bind to a hormone to increase the activity of the hormone. This might occur by protecting the hormone from degradation and increasing the biological half-life or improving the delivery to the target tissue. This has been demonstrated with ST and IGF-1. Antibodies can also be directed to the target tissues to block the receptors of catabolic hormones or stimulate the receptors of anabolic hormones (anti-idiotypic antibodies; see Section 2.4), as has been demonstrated for β-AA. Antibodies can also be developed that destroy cells. An example would be removing fat cells to prevent the accumulation of lipid. Many of these approaches are still under development. Since antibodies are proteins that will be inactivated by cooking, there should be significantly less concern about residues in meat from this immunological approach.

3.8 Dietary Chromium and Insulin

Key concepts

- Chromium has been proposed to be an essential trace mineral that is required for normal carbohydrate and lipid metabolism, but this has not been firmly established.
- The proposed biologically active form of Cr is low-molecular-weight chromium-binding substance (LMWCr) or chromodulin.
- Chromodulin improves insulin action by increasing the phosphorylation of the insulin receptor and the translocation of the GLUT4 transporter to the cell membrane.
- Dietary requirements for chromium may be much higher for livestock species than for healthy humans because of production stressors.
- Chromium supplementation may be beneficial in growing and lactating cattle, pigs, and broilers and layers.
- Organic complexes of chromium have been developed that are more bioavailable.

Chromium in the trivalent form (Cr^{3+}) has been proposed to be an essential trace mineral that is required for normal carbohydrate and lipid metabolism, but this is not without controversy. The idea that chromium was an essential trace mineral began in the mid-1950s with the identification of a glucose tolerance factor (GTF) from yeast. GTF was proposed to be a complex of chromium with niacin, glycine, cysteine and glutamic acid, but this has not been substantiated and it is now suggested that GTF was an artefact of the purification process. Much of the early research on chromium has been questioned and nutritional studies have shown chromium having a beneficial effect only at very high pharmacologically relevant doses (reviewed in Vincent, 2017 and Vincent and Stallings, 2018) or in situations of metabolic stress and impaired insulin function (Anderson, 2013).

The proposed biologically active *in vivo* form of Cr, known as the low-molecular-weight chromium-binding substance (LMWCr) or chromodulin, is now thought to be a low-molecular-weight (1500 Da) oligopeptide that contains four Cr ions per molecule. The peptide consists of glycine, cysteine and a large proportion of glutamate and aspartate, which is critical for Cr binding. However, chromodulin from different sources is somewhat different in composition and it has not yet been sequenced, so its structure has not been confirmed.

The major role proposed for dietary Cr is to increase the sensitivity of cells to insulin and to amplify the insulin response. Cr improves the function of insulin to reverse the effects of clinical hyperglycaemia by increasing the uptake of glucose into tissues for lipogenesis and glycogen synthesis. Cr may also increase the effects of insulin in stimulating the uptake of amino acids and increasing protein synthesis in muscle. Conditions that increase circulating glucose and insulin increase the

excretion of Cr in the urine. The signs of 'chromium deficiency' are related to impaired insulin function and include impaired glucose tolerance, elevated levels of insulin, cholesterol and triacylglycerols in plasma, glucosuria, impaired growth and decreased longevity and fertility. Cr supplementation may be effective in humans receiving parenteral nutrition or with type II (insulin-independent) diabetes, but not with type I diabetes. Cr supplementation is also effective in animals under various forms of stress, such as newly arrived feedlot cattle and first-lactation dairy cows. However, it is not clear in many *in vitro* and dietary supplementation studies if the effects seen are more pharmacologically or physiologically relevant.

Insulin

Insulin is a polypeptide hormone produced by the β cells in the islets of Langerhans in the pancreas. Insulin is secreted from the β cells in response to rising ATP levels produced from increased glucose levels in the β cells. The primary role of insulin is to regulate blood glucose levels by increasing the uptake of glucose into tissues and storage as glycogen or lipid. It also plays a role in promoting amino acid uptake in species where little fluctuation in blood glucose is seen, such as in functional ruminants and carnivores. Insulin acts on cells containing an insulin receptor, especially in adipose tissue, liver and muscle.

The insulin receptor (see Section 1.3) consists of extracellular α-subunits and intracellular β-subunits held together by disulfide bonds. The amino acids in the positions 29–48 of the α-subunit are considered critical for insulin binding, which causes autophosphorylation of the β-subunit and activates its tyrosine kinase. The amino acid sequence of insulin is very similar among vertebrates, with minor changes mainly at positions 8, 9 and 10 of the α-subunit and position 30 of the β-subunit. As a result, insulin isolated from one species is somewhat active in another. Insulin is synthesized as proinsulin, with the active molecule formed from cleavage of the 23 amino acid C peptide (see Fig. 1.11). There is a high degree of species variability in the C peptide sequence.

Insulin acts on the liver to increase glucose uptake and the formation of glucose-6-phosphate. Insulin also activates a phosphatase enzyme that dephosphorylates and activates glycogen synthetase. In adipose tissue, glucose is converted to glycerol and combined with free fatty acids to form triacylglycerol. Lipid synthesis is increased by stimulation of citrate lipase, acetyl-CoA carboxylase, fatty acid synthase and glycerol-3-phosphate dehydrogenase. In muscle, insulin stimulates the uptake of glucose and amino acids and stimulates glycogen and protein synthesis. Protein catabolism is also decreased. Insulin also acts by triggering the exocytosis of vesicles into the plasma membrane, which leads to an increased amount of the glucose transporter GLUT4. Insulin also has a direct vasodilatory effect to increase blood flow and nutrient supply to muscles. Rapid growth and leanness in domestic animals are related to enhanced sensitivity of muscles to insulin and enhanced glycolytic metabolism of muscles. Decreased insulin sensitivity results in increased basal levels of insulin and increased carcass fat with decreased growth rate.

Glucagon

Glucagon is a peptide hormone that is produced in the α-cells of the pancreas islets and acts to increase blood glucose by increasing glycogenolysis or gluconeogenesis. Glucagon is a single-chain polypeptide of 29 amino acids that is identical in sequence across all mammals and highly conserved across all species. It is formed from proglucagon by the prohormone convertase 2 enzyme in the pancreas (see Fig. 3.36). Proglucagon is also processed by the prohormone convertase enzyme 1/3 in the neuroendocrine cells of the intestine to proglucagon-derived peptides (PGDPs) (see Section 3.10), which regulate feed intake.

The effects of glucagon are opposite to those of insulin. In the liver, it stimulates glycogenolysis and gluconeogenesis from amino acids and glycerol to maintain sufficient levels of blood glucose. This is particularly important during prolonged fasting, exercise or during neonatal life. Glucagon also stimulates lipolysis in adipocytes.

There are three other cell types that are part of the pancreatic islets: the γ cells which produce pancreatic polypeptide, the δ cells which produce somatostatin and the ε cells which secrete ghrelin. The secretory activities of the α and β cells are regulated through paracrine and autocrine mechanisms among the different cell types. Hyperglycaemia activates β cells to produce insulin and inhibits α cells, while hypoglycaemia activates α cells to produce glucagon. Pancreatic polypeptide produced by the γ cells can inhibit the release of glucagon. Ghrelin produced by the ε cells can inhibit insulin

and somatostatin secretion. Somatostatin production by the δ cells inhibits or decreases secretion of insulin, glucagon and pancreatic polypeptide. For more information, see the review by Da Silva Xavier (2018).

Potential mechanisms of action of chromium

Chromium chloride ($CrCl_3$) and chromium picolinate (CrPic) are two of the common dietary forms for supplementing Cr. Cr is thought to be transported in the blood bound to the metal transport protein transferrin, Cr-transferrin (Cr-Tf). An increase in plasma insulin increases the number of transferrin receptors at the plasma membrane and these receptors may bind and transport Cr-Tf into the cell. Inside the cell, Cr is transferred to chromodulin.

Chromodulin activates insulin receptor tyrosine kinase, the enzyme that phosphorylates the insulin receptor, and inhibits the phosphatase enzyme leading to increased phosphorylation of the insulin receptor, thus enhancing insulin intracellular signalling. These effects are not seen in the absence of insulin, and chromodulin without bound Cr (apochromodulin) does not affect the insulin receptor tyrosine kinase activity. When insulin levels decrease following glucose uptake, Cr bound to chromodulin is eliminated from the cells and excreted in the urine. Chromodulin is stored in its metal-free apo- form inside insulin-sensitive cells, but the mechanism of regulation of chromodulin levels is not known. Chromodulin is reminiscent of the calcium-binding protein calmodulin (see Section 1.3) in that both proteins activate protein kinases.

A potential mechanism of how Cr potentiates the action of insulin is illustrated in Fig. 3.26. When insulin (I) binds to the α-subunit of the insulin receptor (IR), it autophosphorylates tyrosine residues on the internal portion of its β-subunit and is converted to the active form. Cr uptake via Cr-Tf converts apochromodulin to holochromodulin by binding four Cr ions. This active holochromodulin then binds insulin receptors, and activates the insulin-stimulated receptor kinase activity, increasing the duration and magnitude of insulin signalling. Chromium also improves translocation of glucose transporter 4 (GLUT4) found primarily in the striated and cardiac muscle and in adipose tissue, leading to improved glucose uptake. This increases sensitivity to insulin in affected cells and enhances glucose tolerance. When the insulin concentration drops, holochromodulin is released from the cell and Cr is excreted. Another hypothesis is that Cr^{3+} undergoes oxidation to Cr^{6+}, which inhibits protein tyrosine phosphatase.

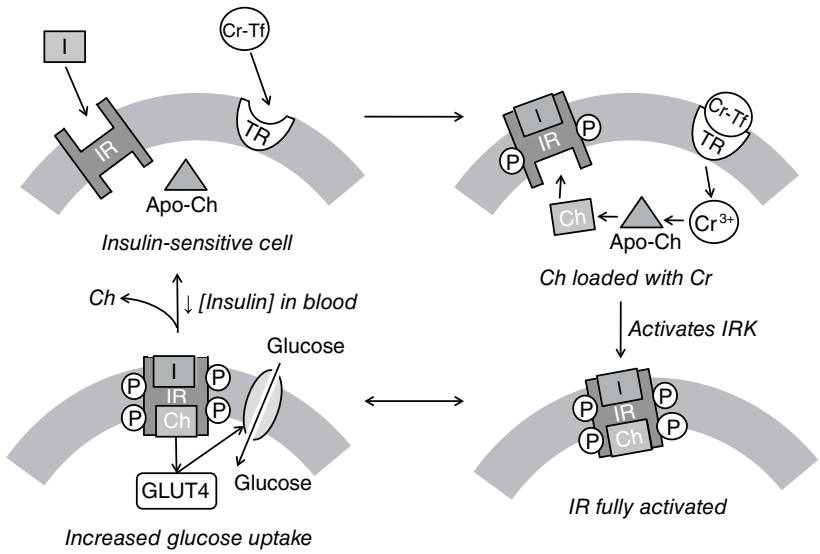


Fig. 3.26. Suggested mechanism for the activation of insulin receptor kinase activity by chromium. TR, transferrin receptor; Cr-Tf, Cr bound to transferrin; I, insulin; Ch, chromodulin; IR, insulin receptor; IRK, insulin receptor kinase; GLUT4, glucose transporter 4.

Detection of glucose levels in plasma by the pancreatic islet cells, as well as uptake and release of glucose by tissues, involves glucose transport proteins (GLUTs) in the cell membrane. These proteins catalyse the transport of glucose down concentration gradients and into target cells. There are five glucose transporter isoforms, GLUT1–4 and GLUTX1 (which is involved in early blastocyst development), along with a fructose transporter, GLUTS.

The GLUTs differ in tissue distribution and kinetic properties and play a key role in glucose homeostasis. GLUT1 is present at high levels in erythrocytes and in endothelial cells lining the blood vessels in the brain. GLUT3 is present in neurons and, together with GLUT1, allows the brain to take up adequate glucose. GLUT2 is a low-affinity transporter that is part of the glucose sensor system in pancreatic cells and is involved in moving dietary glucose across the basolateral membranes of intestinal epithelial cells. GLUT2 is also important in the transport of glucose out of the liver and kidney and into the blood. GLUT4 is the major insulin-responsive isoform found in adipose tissue and striated muscle. It is normally located in intracellular storage compartments and is translocated to the plasma membrane in response to insulin. When insulin levels decline, GLUT4 is recycled back into intracellular storage.

In addition to the increased tyrosine kinase activity, Cr increases the translocation of the GLUT4 from the Golgi apparatus to the cell membrane. This improves glucose uptake in adipose tissue and skeletal muscle. Cr decreases levels of cholesterol in the plasma membrane and improves the cytoskeletal structure involved in translocation of GLUT4. Cr also has been reported to be involved in stabilizing nucleic acids and stimulating RNA and protein synthesis, but the mechanism behind these effects has not been established.

Physiological effects

Many of the proposed beneficial effects of Cr supplementation in the diets of healthy humans have not been well substantiated. These include weight loss, specifically a decrease in body fat and increase in lean muscle mass, which are due to the enhanced action of insulin.

Cr is found in a wide variety of foods, including potatoes, whole grains and brewer's yeast. The response of animals to Cr supplementation of practical diets is variable, since some diets may already have adequate levels of Cr, or the bioavailability of dietary Cr supplement is low. The Cr status of the animals can also vary, so that in some situations the requirement for Cr is increased due to increased excretion of Cr. Thus, diets must be deficient in Cr or the animal must require additional dietary Cr before supplementation of diets with Cr can be effective. Normal levels *in vivo* are very low and it is very difficult to have a deficiency of Cr under normal conditions.

Some livestock species, such as pigs and broiler chickens, grow at incredibly fast rates, with increases in body weight of 60- and 80-fold over birth weight to market weight for broilers and pigs, respectively. Livestock animals are also subjected to a variety of stress conditions associated with reproduction, lactation and production conditions (weaning, crowding, transportation, disease challenges, etc.) that affect animal health and performance. Thus, dietary requirements of Cr may be much higher for livestock species than for healthy humans. Cr supplementation of livestock has been shown to reduce serum cortisol, alter serum lipid and cholesterol profiles, increase fecundity and improve growth performance under stressful conditions. The potential physiological responses of commercial animals to Cr are summarized in Fig. 3.27.

Cr supplementation for cattle may be of benefit under stressful conditions, such as transportation and regrouping of animals and metabolic stress of lactation. Supplementing newly arrived feedlot cattle with dietary Cr is reported to decrease morbidity and lower plasma cortisol but the response is variable. The immune response is also improved, as measured by the blastogenic response of peripheral blood mononuclear cells cultured with T-lymphocyte mitogens. The beneficial effects of Cr were not seen when calves were given a long-acting antibiotic shortly after arriving at the feedlot. Cr increases glucose utilization in growing heifers and is effective in first-lactation dairy cows in the transition period but not in multiparous cows. Supplemental Cr given through late gestation and into the first weeks of lactation tends to improve milk yield in the first 4–6 weeks of lactation. The metabolic stress during transition is also lowered, with decreased levels of blood ketone bodies. There is also improved cell-mediated immune response in lactating cows via changes in production of cytokines. The specific role of Cr in cattle undergoing stress has not been established.

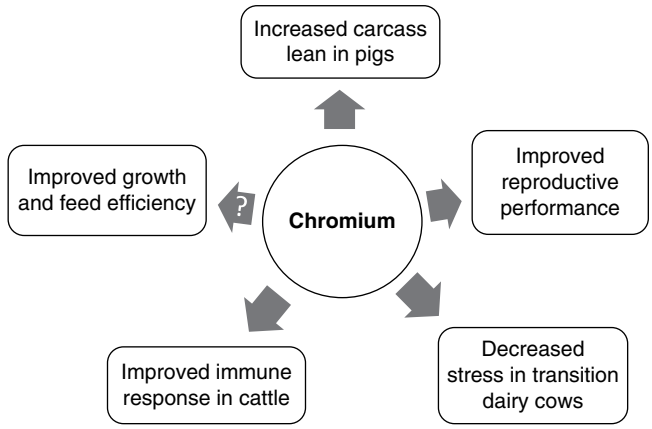


Fig. 3.27. Potential physiological responses to chromium.

Cr supplementation is not effective in young weaning pigs but has shown benefits in older growing and lactating pigs. Cr supplementation has been shown to alter metabolism in pigs to improve feed efficiency and growth rate, increase lean muscle mass and decrease fat content in carcasses, and improve reproductive performance.

Supplemental Cr has been reported to reduce mortality and improve the plasma lipid profile in broiler chickens but consistent effects on growth rate, feed efficiency and carcass fat were not found. Cr improved the performance of turkey poults under disease stress and broiler chicks under low temperature stress. Layer chickens and broiler breeder hens supplemented with Cr had lower serum and egg yolk cholesterol.

Research with rabbits has shown that the cholesterol and plaque content in the aorta is decreased with supplemental Cr. Cr supplementation has also been reported to alter glucose metabolism in fish and to increase weight gain, energy disposition and liver glycogen. For a comprehensive review of the role of Cr in animal nutrition, see Lindemann and Lu (2018).

Dose

In the USA, chromium picolinate and chromium propionate are approved for use in pigs up to 200 ppb and chromium methionine is approved for use up to 400 ppb. Chromium yeast is approved in Canada for use in first-lactation heifers up to 400 ppb. For humans, a daily adequate intake of Cr of 35 μg day^{-1} for an adult male and 25 μg day^{-1} for an adult female is recommended by Figueiredo *et al*. (2020).

Trivalent chromium has been used as a dietary supplement in the form of chromium picolinate (CrPic), chromium nicotinate (CrNic) and high-chromium yeast, which are absorbed more efficiently than inorganic chromium chloride. The efficacy of dietary Cr may be affected by inefficient absorption of Cr, particularly if it is not complexed with an organic molecule such as picolinate. Absorption is increased in diabetes, possibly because diabetics are deficient in Cr. The absorption of Cr is inversely related to dietary intake, ranging in humans from 2% absorption at 10 μg day^{-1} in the diet to 0.5% absorption with > 40 μg day^{-1} in the diet. At a dose of 1000 μg day^{-1}, only 0.4% of chromium chloride is absorbed, while chromium picolinate is absorbed at rates as high as 2.8%. Other organic complexes of Cr have been developed, such as chromium (D-phenylalanine)$_3$ (Fig. 3.28) and the trinuclear cation $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$; the latter mimics the ability of chromodulin to increase the tyrosine kinase activity of the insulin receptor but is more stable than chromodulin and is orally active. It is absorbed at a more than tenfold higher rate than chromium picolinate.

Factors affecting the bioavailability of dietary Cr in feeds and the relative bioavailability of Cr from different supplements are not well understood. The low bioavailability of inorganic Cr may be due to the formation of insoluble chromic oxide, binding to chelating agents in feeds, and slow conversion to the biologically active form. Measurements of the total Cr content of feeds are therefore poorly

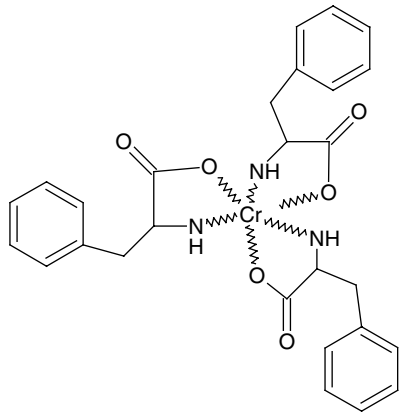


Fig. 3.28. Structure of chromium phenylalanine $Cr(D\text{-}Phe)_3$.

related to the amount of biologically active Cr. The absorption of Cr is increased by vitamin C, amino acids or oxalate. Alternatively, intake of antacids, high fibre, phytate and simple sugars will reduce absorption. The kidney, liver and spleen contain the highest amounts of Cr in the body.

Plasma levels of Cr range from 0.12 to 0.67 μg l^{-1} and are lower during infections and after glucose loading. Turnover of Cr follows a three-compartment model with half-lives of 0.5, 6 and 83 days. Plasma levels of Cr are not a good indicator of tissue Cr levels, since these different Cr pools do not seem to be in equilibrium. Cr is excreted mostly in the urine as chromodulin, with 24 h excretion rates of 0.22 μg day^{-1} in humans. Injection of chromodulin leads to increased excretion of Cr, as chromodulin is poorly reabsorbed in the kidney. Excretion of Cr increases after glucose loading and is higher in diabetics. Stress and exercise also increase Cr excretion, with several times more Cr excreted after extreme trauma. Uptake of Cr by tissues decreases with age. There is a correlation between low circulating levels of Cr and the incidence of type II diabetes.

Safety issues

Cr toxicity is primarily associated with exposure to hexavalent Cr^{6+} salts, which are strong oxidizing agents used in alloying, tanning and in the production of corrosion-resistant paints. Workers exposed to these compounds suffer from dermatitis, cancer, gastroenteritis, nephritis and hepatitis. Hexavalent Cr^{6+} is more soluble, more readily absorbed and is at least five times more toxic than Cr^{3+}. The LD50 for Cr^{3+}–nicotinic acid complex injected intravenously is 60 mg kg^{-1} BW in rats. The lethal single dose of Cr^{6+} in young rats is 130 mg kg^{-1} BW, while as much as 650 mg of Cr^{3+} kg^{-1} BW produced no toxicosis. There is some evidence that chromium picolinate may be harmful, since release of Cr from chromium picolinate requires reduction of the chromic centre, which may lead to the production of harmful hydroxyl radicals. There is no information on Cr toxicity in pigs. Feeding a diet containing 2000 mg $CrCl_3$ kg^{-1} decreased growth rate of chicks, while levels of 1000 mg kg^{-1} had no effect on growth. The concentrations of trivalent chromium that are typically added as dietary supplements for most food-producing animals are assumed safe and non-toxic. Because of the low absorption of Cr, toxic effects from dietary Cr are rarely found.

Analytical methods for Cr have been problematic, so studies before 1980 are questionable. Cr levels in tissues are very low and early analytical methods were not sensitive enough; there was also contamination of samples with Cr, for example from stainless steel needles and homogenizer blades.

In conclusion, the current hypothesis suggests that interactions between Cr and insulin are mediated by the amount of Cr bound to chromodulin, which then stimulates phosphorylation and activity of the insulin receptor. This interaction also increases the amount of GLUT4 in the plasma membrane, as does increasing membrane fluidity through removing cholesterol to improve glucose transport and utilization. This is an important area of research that requires further study with a potential to have significant impacts on animal production and human health.

3.9 Dietary Polyunsaturated Fatty Acids

Key concepts

- PUFAs regulate the expression of genes involved in lipid and carbohydrate metabolism.
- PUFAs act either directly by binding nuclear receptors or indirectly by affecting the levels of transcription factors in the nucleus.
- PUFAs can be metabolized to eicosanoids which act through cell surface G protein-coupled receptors.
- Short-chain *n*-3 and *n*-6 PUFAs undergo competitive elongation and desaturation reactions to

form longer-chain PUFAs that can be metabolized to eicosanoids.

- *N*-3 and *n*-6 PUFAs have opposing physiological functions.
- *N*-3 PUFA, GLA and CLA have beneficial health effects.
- *Trans* fatty acids generated by industrial partial hydrogenation of vegetable oils can have negative effects on metabolism and health.

Polyunsaturated fatty acids (PUFAs) are important components of cell membranes that influence membrane function, signal transduction, eicosanoid metabolism and gene expression. The number and position of the double bonds in the fatty acid (for example 18:2, *n*-3 means an 18-carbon fatty acid with two double bonds starting at carbon 3, counting from the methyl end of the molecule) has dramatic effects on its biological activity. Also important is whether the double bond is in the *cis* or *trans* configuration; a *cis* double bond introduces a kink in the carbon chain, while the *trans* configuration does not (see Fig. 3.31). Animals cannot insert double bonds into fatty acids beyond carbon 9 from the carboxyl end of a fatty acid. Thus, both linoleic acid (LA) (18:2, *n*-6) and α-linolenic acid (ALA) (18:2, *n*-3) are essential PUFAs that must be obtained from the diet. These fatty acids are converted through desaturation and elongation reactions to longer-chain *n*-3 and *n*-6 fatty acids.

Mechanism of action

PUFAs and their metabolites are involved in regulating the expression of enzymes involved in lipid and carbohydrate metabolism (Fig. 3.29). PUFAs cause decreased lipid synthesis in the liver, increased fatty acid oxidation in the liver and skeletal muscle, and increased synthesis of glycogen. Thus, PUFAs act as repartitioning agents, increasing the oxidation of fatty acids rather than storage as triacylglycerols and increasing glucose storage as glycogen.

PUFAs are also involved in the control of adipogenesis. Activation of adipocyte genes leading to adipocyte differentiation is stimulated by a metabolite of PGD_2 (15-deoxy-prostaglandin J2) interacting with the peroxisome proliferator-activated receptor γ (PPARγ). Adipocyte differentiation is inhibited by $PGF_{2\alpha}$, which interacts with a cell surface receptor to alter intracellular calcium levels. The balance between these two pathways determines the overall effect of PUFA metabolites on adipocyte differentiation.

Fatty acids regulate gene expression by binding nuclear transcription factors, including PPARα, β and γ, hepatic nuclear factor (HNF4α) and nuclear factor-Y (NF-Y). Fatty acids bind to the PPAR, and the complex binds to responsive elements (PPRE) on DNA as a heterodimer with 9-*cis* retinoic acid receptor (RXR) to increase gene expression (see Section 1.3). PPARα is activated by lipid catabolic

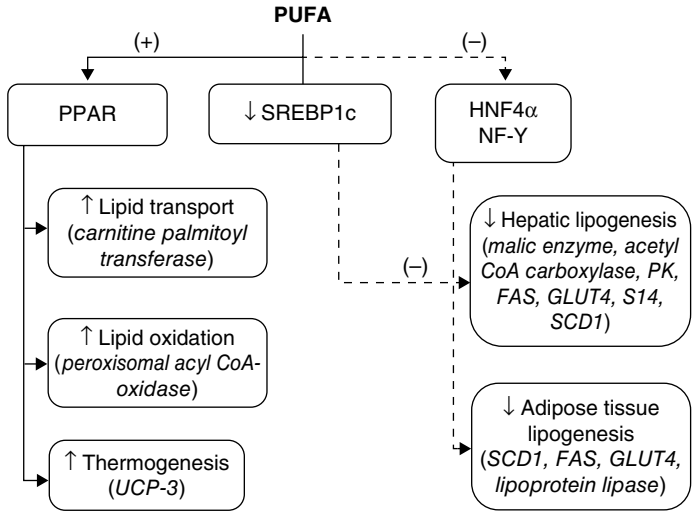


Fig. 3.29. Metabolic effects of dietary PUFA. PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; HNF, hepatic nuclear factor; NF-Y, nuclear factor-Y; PK, pyruvate kinase; FAS, fatty acid synthase; GLUT4, glucose transporter 4; SCD, stearoyl-CoA desaturase; UCP, uncoupling protein.

fibrate drugs and PUFAs (especially long-chain *n*-3 PUFAs, such as eicosapentaenoic acid (EPA)) to increase the expression of genes involved in lipid transport, oxidation and thermogenesis, including carnitine palmitoyltransferase, peroxisomal acyl-CoA oxidase, cytochrome P4504A and uncoupling protein 3.

Fatty acyl-CoA also binds to HNF-4α and NF-Y, which decreases their DNA binding activity and decreases the expression of genes involved in lipoprotein metabolism. PUFAs also inhibit hepatic lipogenesis by decreasing the expression of enzymes involved in glucose metabolism and fatty acid biosynthesis. Malic enzyme, acetyl-CoA carboxylase, L-Type pyruvate kinase, fatty acid synthase (FAS), GLUT4, S14 protein and stearoyl-CoA desaturase (SCD1) in the liver are decreased by PUFAs. The expressions of SCD1, FAS, GLUT4 and lipoprotein lipase in adipose tissue are also decreased by PUFAs. The suppression of lipogenesis occurs through decreasing levels of the transcription factor sterol regulatory element-binding protein (SREBP1c) in the nucleus. High levels of fatty acyl-CoA decrease the processing of SREBP1c from the endoplasmic reticulum and Golgi to the nucleus, thus decreasing the expression of genes involved in lipogenesis, fatty acid elongation and desaturation, and very-low-density lipoprotein (VLDL) assembly. Decreased levels of SREBP1c in the nucleus also decrease the *trans*-activation of NF-Y responsive genes. PUFAs can affect both the level of gene transcription and mRNA stability. PUFA can also be metabolized to the eicosanoids, which activate cell-surface G protein-coupled receptors, which results in intracellular signalling cascades and subsequent activation of transcription factors. For further details, see the review by Saini and Keum (2018).

Linoleic acid, linolenic acid and γ-linolenic acid

Linoleic acid (LA) (18:2, *n*-6) and α-linolenic acid (ALA) (18:3, *n*-3) are essential dietary PUFAs that are converted by elongation and desaturation reactions to other *n*-6 and *n*-3 fatty acids, respectively. LA is ultimately metabolized to arachidonic acid (AA), which is the precursor of 2-series prostaglandins and 4-series leukotrienes (see Section 1.2). Dihomo-γ-linolenic acid (DGLA), an intermediate in the formation of AA, is converted into 1-series prostaglandins (PG) and 3-series leukotrienes (LT). ALA is metabolized to eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA). EPA is converted into the 3-series prostaglandins and 5-series leukotrienes. This is summarized in Fig. 3.30.

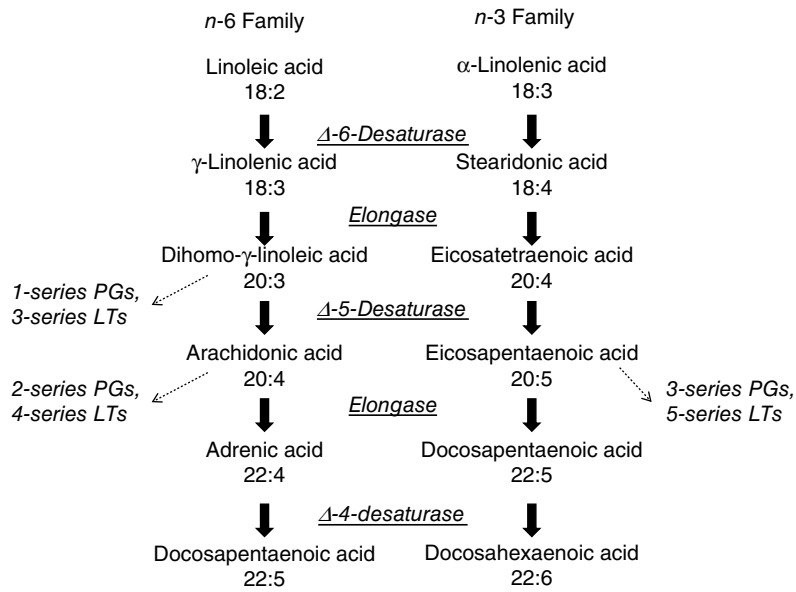


Fig. 3.30. Metabolism of *n*-3 and *n*-6 fatty acids.

Changes in the amounts of the *n*-3 and *n*-6 fatty acids can alter the structural properties of cell membranes, such as fluidity and permeability. This can affect the activity of ion channels and membrane proteins. In addition, the availability of 20-carbon precursors for the synthesis of prostaglandins and leukotrienes can have dramatic endocrine effects. The Δ6 desaturase enzyme catalyses the conversion of LA to γ-linolenic acid (GLA) by adding a *cis* double bond at the 6th carbon from the carboxyl end of LA (Fig. 3.31); this is the rate-limiting step in the *n*-6 reaction cascade. The activity of the Δ6 desaturase is increased by insulin and inhibited by epinephrine, cortisol, thyroxine, glucagon, saturated fat and ageing. Thus, dietary supplementation with GLA may be necessary to provide sufficient precursors for the synthesis of DGLA and the series-1 prostaglandins. GLA is found in natural plant oils, including evening primrose oil (9%), borage oil (23%) and blackcurrant oil (18%).

The *n*-3 and *n*-6 PUFAs have opposing physiological functions and their balance is important for normal growth and development. The *n*-6 and *n*-3 pathways share the same enzymes, so an increase in LA results in an increase in AA and a decrease in the formation of the *n*-3 fatty acids, EPA and DHA. Along with the total amount of fatty acids available, the *n*-6:*n*-3 ratio affects the formation of the long-chain PUFAs by competition for the same enzymes and transport systems. The affinity of the enzymes is higher for the *n*-3 fatty acids, and efficient conversion of ALA to long-chain *n*-3 PUFAs can occur at a 4:1 ratio of LA to ALA in the diet. Since the longer-chain *n*-3 PUFAs are the most biologically active, dietary supplementation with DHA and EPA is necessary if the metabolism of ALA is impaired. Good dietary sources of DHA and EPA are various marine oils, while ALA is found in flax, canola (rapeseed), perilla and soybean oils.

The *n*-3 fatty acids have been implicated in the prevention and management of coronary heart disease and hypertension. The *n*-3 fatty acids stabilize myocardial membranes electrically, resulting in decreased susceptibility to ventricular arrhythmia and reduced risk of sudden death. DHA is essential for growth and functional development of the brain in infants, and deficiencies of DHA are associated with many types of mental disease. The *n*-3 fatty acids have potent anti-inflammatory effects, and high doses of *n*-3 fatty acids can reduce plasma triacylglycerol levels.

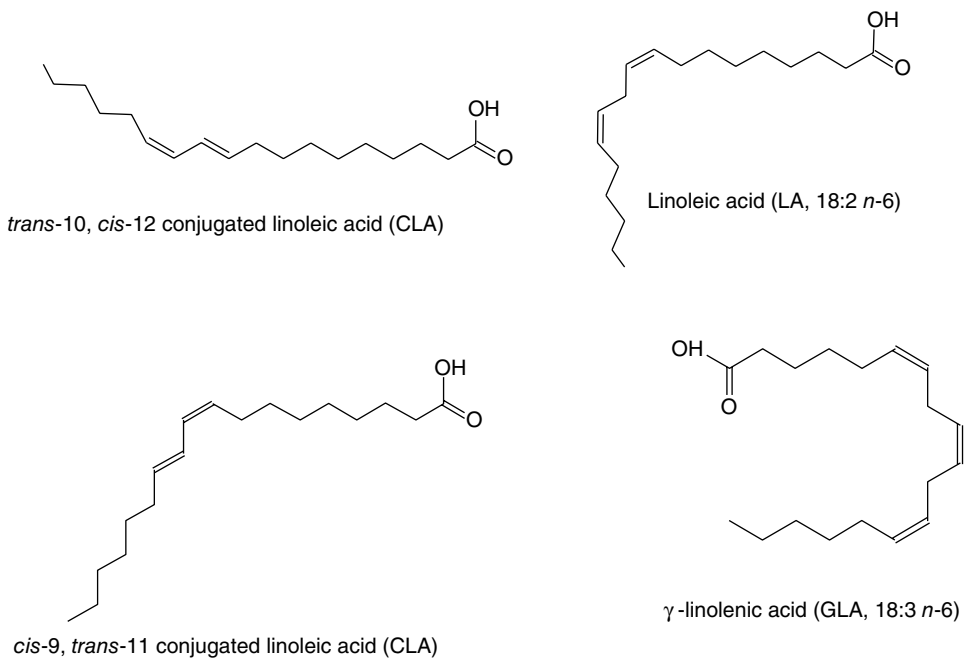


Fig. 3.31. Structures of linoleic acid, conjugated linoleic acid and γ-linolenic acid.

As the levels of DHA and EPA increase, the levels of AA decrease in cell membranes of platelets, erythrocytes, monocytes, neutrophils and liver cells. This decreases the levels of the eicosanoids produced from AA that enhance platelet aggregation, immunosuppression and the development of inflammation and allergic reactions. These include prostaglandin E_2 (PGE_2), thromboxane A_2 (a potent platelet aggregator and vasoconstrictor), leukotriene B_4 (an inducer of inflammation, leukocyte adherence and chemotaxis) and thromboxane A_3 (a weak platelet aggregator and vasoconstrictor). There is also increased production of prostacyclin PGI_3 and maintenance of PGI_2 (both active vasodilators and inhibitors of platelet aggregation), increased levels of leukotriene B_5 (a weak inducer of inflammation and chemotaxis) and production of potent anti-inflammatory resolvins and protectin. Thus, an increase in the formation of eicosanoids from AA increases the formation of blood clots, increases blood viscosity and vasoconstriction and increases inflammation.

The appropriate balance of *n*-6:*n*-3 fatty acids in the diet is important. Deficiencies of the *n*-6 fatty acids have been linked with depressed growth, impaired immune function, cardiovascular disease, diabetes and cancer in humans. The diets of Western countries have high levels of LA, which has been promoted for its cholesterol-lowering effects. Deficiencies in longer-chain DHA and EPA were considered to be unlikely, except under conditions where there is decreased activity of the Δ6 desaturase enzyme. However, the conversion of ALA to DHA is about 1% in infants and even lower in adults. Supplementation of diets with ALA results in small increases in EPA but no effect on DHA, so direct supplementation with EPA and DHA is recommended. For more information, see Rodríguez *et al*. (2019).

Supplementation with GLA, an *n*-6 PUFA that has the same number of double bonds as ALA, has similar physiological effects to supplementing with *n*-3 PUFA. GLA supplementation increases tissue levels of DGLA and the 1-series prostaglandins, particularly PGE_1, which has anti-inflammatory and immunoregulating properties. Levels of AA are not increased, since the levels of the Δ5 desaturase are low. DGLA competes with AA for the cyclooxygenase enzyme and thus further lowers the synthesis of the pro-inflammatory series-2 prostaglandins from AA. GLA supplementation also lowers blood pressure and plasma cholesterol. GLA supplementation has also been shown to decrease the accumulation of body fat in rats.

Applications

In non-ruminants, dietary fatty acids are absorbed unchanged and can be incorporated into tissue lipids. Thus, dietary lipids can be used to modify the fatty acid composition of pig and poultry products directly. In contrast, rumen microorganisms modify dietary lipids by isomerization, hydrolysis and hydrogenation reactions, to form novel fatty acids including conjugated linoleic acid (CLA) (see below).

Supplementing diets with *n*-3 fatty acids may be effective in treating metabolic diseases of poultry, including ascites and sudden death syndrome (heart attack) in broilers and turkeys. Ascites is caused by an imbalance between the oxygen requirements of the bird for growth and metabolism, and the delivery of oxygen to the tissues, resulting in tissue hypoxia. This increases the haematocrit and blood viscosity, which leads to pulmonary hypertension and hypertrophy of the right ventricle of the heart. Eventually, the atrioventricular valve fails, causing back pressure and ascites fluid leakage from the liver. Supplementing broiler diets with 5% flax oil decreases blood viscosity and reduces the incidence of ascites. The addition of flaxseed, flax oil or other sources of *n*-3 fatty acids to layer rations reduces the accumulation of fat in the liver of laying hens. In addition, supplementing animal diets with *n*-3 fatty acids not only improves the health of the animals but also provides a good dietary source of *n*-3 fatty acids for human consumption.

A major limitation to supplementing animal diets with PUFAs is the potential for increased lipid peroxidation and rancidity; these problems have been at least partly addressed by increasing levels of vitamin E and other antioxidants. The *n*-3 fatty acids can also lead to 'fishy' odours and taste. Eggs enriched with *n*-3 are now produced commercially by including flax in layer rations. Cow's milk enriched with DHA is produced by the addition of a DHA-rich feed additive to dairy feed rations. This additive contains a marine source of DHA and a bypass ingredient that protects the DHA from hydrogenation in the rumen, so that a significant amount of DHA accumulates in the milk. Research on enriching the *n*-3 content of meat from poultry, pigs and cattle without adversely affecting the stability and organoleptic properties is continuing. For a review, see Butler (2014).

Trans fatty acids

The PUFAs described so far have double bonds in the *cis* configuration, while *trans* fatty acids are unsaturated fatty acids that contain one or more double bonds in a *trans* configuration. Some *trans* fatty acids in food are produced during rumen fermentation and these have beneficial health effects (see below). However, most of the *trans* fatty acids in food products are generated by industrial partial hydrogenation of vegetable oils rich in PUFAs and these have negative effects on metabolism and health.

The content of *trans* fatty acids in partially hydrogenated vegetable oils can be as high as 60%, mostly due to *trans*-octadecenoic acid (*trans* 18:1). In the *trans* configuration, the hydrogen atoms around the double bond point in opposite directions, while in the *cis* configuration the hydrogen atoms point in the same direction. This creates a kink in the alkyl chain of the *cis* form, while in the *trans* form the bond angles correct each other to produce a straighter chain that is similar to saturated fatty acids (see Fig. 3.31). This allows *trans* fatty acids to pack together more tightly than *cis* fatty acids, which raises their melting point to improve texture, taste and shelf life of food products. However, this structure also affects their physiological properties and there is a positive association between the intake of industrial *trans* fatty acids and the development of cardiovascular diseases. Industrial *trans* fatty acids have negative effects on lipid metabolism, inflammation, oxidative stress, endoplasmic reticulum (ER) stress, autophagy and apoptosis, while *cis* fatty acids are protective. This has led to restrictions on the use of *trans* fatty acids in food products.

Trans fatty acids negatively affect the overall plasma lipoprotein profile, to increase plasma LDL cholesterol and total cholesterol and decrease HDL cholesterol. They also stimulate inflammation via NF-κB signalling to induce the expression of pro-inflammatory cytokines, including TNFα, IL6 and IL1B, and promote oxidative and ER stress. This impairs ER function and stimulates the unfolded protein response (UPR), which improves protein folding to promote cell survival but can lead to apoptotic cell death if the load of unfolded proteins is not reduced. An imbalance between production of reactive oxidant species (ROS) and antioxidant defence can result in oxidative stress and subsequent damage to lipids, DNA and protein. Autophagy is also induced whereby organelles and other intracellular components are degraded in the lysosome to provide energy for the cell. In the liver, industrial *trans* fatty acids stimulate the expression of cholesterol and fatty acid synthesis genes and activate the expression of sterol regulatory element binding protein (SREBP). This increases fat storage in the liver and can lead to non-alcoholic fatty liver disease (NAFLD). Ruminant *trans* fatty acids show similar effects on human plasma lipoproteins but do not promote inflammation, ER stress and cholesterol synthesis. For more information on *trans* fatty acids, see the review by Oteng and Kersten (2020).

Conjugated linoleic acid

Conjugated linoleic acid (CLA) refers to a group of isomers of linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid), the most biologically important being *cis*-9, *trans*-11 CLA (also known as rumenic acid) and *trans*-10, *cis*-12 CLA (see Fig. 3.31). The US Food and Drug Administration labelling requirements defines *trans* fats as 'all unsaturated fatty acids that contain one or more isolated double bonds in a *trans* configuration'. CLA is not included, since it contains conjugated and not isolated *trans* double bonds. Moreover, CLA has been approved as generally recognized as safe (GRAS) for a mixture of approximately 60–90% of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers.

CLA has a broad range of biological activities, including anti-cancer effects, regulation of energy partitioning and nutrient metabolism, reduction of the catabolic effects of immune challenge, and reduction in blood lipids and prevention of atherosclerosis. Many of these effects have been demonstrated in animal models and have not yet been conclusively shown in humans. From an animal production point of view, CLA acts as an effective repartitioning agent in pigs, to increase feed efficiency, decrease back fat and increase lean in the carcass.

CLA was first isolated from grilled beef and can be formed by heating linoleic acid in the presence of a base. CLA is produced biologically by the biohydrogenation of linoleic and linolenic acid by rumen microbes, and various CLA isomers are found in milk, cheese and beef. The *cis*-9, *trans*-11 isomer is the predominant form of CLA produced by rumen microbes. Most feeding trials with animals use a mixture of *cis*-9, *trans*-11 CLA and

trans-10, *cis*-12 CLA in approximately equal amounts, with other isomers at considerably lower levels. Some commercial preparations may contain isomers with conjugated double bonds at carbon 8, 10, 11 or 13, and this may affect the biological activity. *Trans*-vaccenic acid (*trans*-11-C18:1) is the major *trans* fatty acid of rumen fermentation. It can be converted in mammalian tissues to *cis*-9, *trans*-11 CLA by the Δ9 desaturase enzyme.

Metabolic effects of CLA isomers

Dietary CLA increases feed efficiency and decreases body fat, with a lesser increase in lean content. CLA at 0.5% in the diet of mice decreased body fat by approximately 60% and increased whole-body protein. Results in pigs and poultry have been less dramatic. There is a greater reduction in back fat of pigs of a fatter genotype than lean commercial genotypes. Pigs fed diets containing CLA tended to have about a 6% improvement in feed efficiency and 6% less subcutaneous fat but a 7% increase in intramuscular fat and 2.5% more lean, with no difference in rate of gain compared with controls. There are some negative effects on consumer perceptions on flavour (–3%), juiciness (–12%) and tenderness (–2.5%). In chickens, CLA supplementation reduced abdominal fat content but also reduced feed intake and growth rate.

In mice, the decrease in body fat has been attributed to the *trans*-10, *cis*-12 CLA isomer. This CLA isomer reduces lipoprotein lipase and decreases intracellular levels of triacylglycerol and glycerol in adipocytes. It also reduces the expression of stearoyl-CoA desaturase in adipocytes and depresses milk fat synthesis in cows. CLA also increases carnitine palmitoyltransferase, which is a rate-limiting enzyme in the β-oxidation of fatty acids. CLA also enhances norepinephrine-induced lipolysis and hormone-sensitive lipase activity.

Trans-10, *cis*-12 CLA also reduces the secretion of apolipoprotein B and triacylglycerol in the human hepatoma HepG2 cell line, which would explain the role of CLA in the prevention of atherosclerosis. CLA supplementation in rabbits reduces LDL cholesterol and the incidence of aortic plaques and does not affect HDL cholesterol.

The *trans*-10, *cis*-12 CLA isomer is also probably responsible for many of the effects of CLA on the immune system, including enhancing the immune system, reducing the catabolic effects of immune stimulation and reduced production of PGE_2 and leukotriene B4. CLA appears to shift the immune response from a T_H2-type response (allergic reactions) to a T_H1-type response (cell-mediated functions; see Section 6.3).

The *cis*-9, *trans*-11 isomer of CLA is an effective inhibitor of carcinogenesis. Fish oil is usually required at about 10% of the diet to exert a beneficial effect, while CLA is effective at levels of 1%. The anti-carcinogenic effect of CLA may be due to a decrease in the production of PGE_2 and an increase in tissue levels of retinol.

The CLA-induced increase in growth in young rodents may be due to *cis*-9, *trans*-11 CLA and this may be blocked by the *trans*-10, *cis*-12 CLA isomer. The *cis*-9, *trans*-11 CLA is a potent and high-affinity ligand for the peroxisome proliferator-activated receptor α (PPARα) (see above). PPARα is expressed in liver, skeletal muscle, spleen, kidney and brown adipose tissue and is involved in increasing lipid catabolism and reducing fatty acid synthesis. CLA supplementation produces anti-diabetic effects, including lower plasma glucose, insulin and free fatty acids.

It is apparent that the different isomers of CLA have different biological properties. It is thus essential that the isomer composition of commercially available preparations of CLA be known. Early preparations of CLA, produced by alkali isomerization during the first half of the 1990s, consisted of approximately 20–40% of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, with the remainder consisting of other positional isomers. The CLA products available, starting in 2000, are almost entirely the two desired isomers. The different CLA isomers can be measured as fatty acid methyl esters by gas chromatography or HPLC using silver-impregnated columns.

Dietary CLA supplementation increases the CLA content of pig adipose tissue, with 46% of dietary *cis*-9, *trans*-11 CLA being incorporated, while other isomers are incorporated less efficiently. Thus, CLA levels in pork can be increased to levels similar to those found in dairy products and beef (2–24 mg g^{-1} of fat). Fortification of cow's milk with CLA is hampered by the dramatic decrease in milk fat caused by supplementation of diets with CLA. This is due to a decrease in fatty acid synthesis and desaturation of fatty acids by CLA. However, feeding fresh pasture or supplementing diets with low levels of fish oil or sunflower oil increases levels of *cis*-9, *trans*-11 CLA in both milk and meat.

Mechanism of action of CLA

The effects of CLA may be due to alterations in eicosanoid metabolism and reduced synthesis of PGE_2. The isomers of CLA are elongated and desaturated in the same manner as linoleic acid to produce CLA-derived eicosanoids. This can reduce the conversion of arachidonic acid into PGE_2 and thereby alter eicosanoid signalling pathways. Reduced levels of prostaglandins, such as PGE_2, will decrease the synthesis and action of tumour necrosis factor-α (TNF-α) and interleukin 1 (IL-1). These cytokines induce the inflammatory response in immune cells, catabolism in skeletal muscle and changes in cell-surface proteins and increase the production of the intestinal peptide cholecystokinin, which induces anorexia (see Section 3.10). TNF-α is a key mediator in many chronic pathological conditions, including cachexia (physical wasting), atherosclerosis, carcinogenesis and obesity. Some of the effects of CLA may be due to reduced activity of TNF-α. For more information on the action of CLA, see Kim *et al.* (2016).

3.10 Regulation of Food Intake

Key concepts

- Controlling food intake is important for optimum performance, health and reproductive efficiency.
- The appetite control centres in the arcuate nucleus (ARC) region of the hypothalamus process adiposity and satiation signals to regulate feed intake and energy expenditure.
- Adiposity signals (leptin and insulin) are related to the size of body fat stores.
- Satiation signals from the GI tract (ghrelin, cholecystokinin, GLP-1, oxyntomodulin and the PP-fold peptides) affect energy intake, meal size and motility of the GI tract.
- The ARC produces orexigenic hormonal signals that increase feeding and anorexigenic ones that decrease feeding.
- Synthetic analogues of many of these hormonal signals have been developed to control feed intake.

Introduction

The capacity of animals for growth and production is limited to some extent by the amount of feed they consume, so methods for increasing appetite should increase weight gain and shorten the time taken to reach slaughter weight. This can be particularly important for ruminants on pasture-based systems, where bulky and less nutrient-dense diets have to support the metabolic demands of pregnancy, lactation and growth. Improving feed intake in weaning pigs is important to improve growth and reduce post-weaning diarrhoea. On the other hand, commercial broiler breeders do not regulate their feed intake based on their energy needs, and they must be feed-restricted to maintain optimum body weight, health and reproductive efficiency. Strategies are also needed to control feed intake of pregnant sows, since hyperphagia occurs before there is an increased demand of nutrients for the developing fetus.

Energy balance in an organism is achieved by the coordinated regulation of feed intake and energy output. These are affected by: (i) environmental cues, such as feed availability and composition, photoperiod, temperature and stressors; and (ii) internal signals that measure or respond to the nutritional state and energy level of the organism, including hormones, energy stores and nutrient and metabolite levels. The hormonal signals regulating feed intake include orexigenic signals that increase feeding and anorexigenic ones that decrease feeding (Table 3.5). An additional distinction is between 'satiation signals', such as ghrelin, CCK GLP-1, oxyntomodulin and the PP-fold peptides which affect energy intake, meal size and motility of the GI tract, and 'adiposity signals', such as leptin and insulin, which are related to the size of body fat stores. Satiation and adiposity signals interact with other factors in the brain to control appetite and body weight.

Information is transmitted to the appetite control centres in the arcuate nucleus (ARC) of the hypothalamus. This can occur by stimulation of the vagus nerve, which is sensitive to mechanical distension of the lumen or gut contraction and the chemical properties of the gut contents, as well as hormonal signals. Gastric distension is detected by specialized mechanoreceptors on the vagus nerve ends in the myenteric plexus and in the outer smooth muscle cells of the stomach. The nutrients in the gastrointestinal tract have differential effects on food intake, with triglycerides much less effective than long-chain fatty acids and unsaturated fatty acids more effective than saturated fatty acids. Hormonal signals from the pituitary gland, adipose tissue and gastrointestinal tract (stomach/abomasum, intestine and

Table 3.5. Main orexigenic and anorexigenic signals.

Orexigenic signals	Anorexigenic signals
Neuropeptide Y (NPY)	Pro-opiomelanocortin (POMC)/α-MSH
Agouti-related peptide (AgRP)	Cocaine- and amphetamine-related transcript (CART)
Melanin-concentrating hormone (MCH)	CRH, urocortin (see Section 6.3)
Orexins/hypocretins	Leptin (see Section 3.5)
Galanin	Insulin (see Section 3.8)
Ghrelin (see Section 3.4)	Serotonin
Endocannabinoids	Peptide tyrosine tyrosine (PYY)
Excitatory amino acids (glutamate)	GABA
Endogenous opiates	Glucagon-like peptide-1 (GLP-1)
Catecholamines	Cholecystokinin (CCK)
	Octadecaneuropeptide
	Oestradiol
	Oxyntomodulin (OXM)
	Pancreatic polypeptide (PP)
	Neurotensin

pancreas) (Fig. 3.32) can also feed back directly to the ARC. The ARC is also sensitive to local levels of glucose, some long-chain fatty acids and some amino acids. These nutrients are thought to affect the activity of two serine/threonine kinases, AMPK (AMP activated protein kinase) and mTOR (mammalian target of rapamycin), which regulate the rate of cellular metabolism, protein synthesis, growth and proliferation, based on nutrient availability. For more information, see the review by Cifuentesb and Acostaa (2022).

Central nervous system hormones

The ARC contains two types of neurons that integrate signals of nutritional status to influence energy homeostasis. The medial part of the ARC expresses orexigenic NPY and AgRP and is anabolic, stimulating food intake and energy storage. The lateral part of the ARC expresses the anorexigenic POMC (as a precursor for α-MSH) and CART and is catabolic to inhibit food intake and energy storage. Differences in the production of these neuropeptides affect feeding behaviour and energy expenditure, thereby maintaining energy homeostasis and body weight.

The ARC neurons synapse with neurons in the lateral hypothalamus (LaH), paraventricular nucleus (PVN), dorsomedial nucleus (DMN) and ventromedial nucleus (VMN). The LaH contains two sets of neurons expressing either melanin-concentrating hormone (MCH) or orexins/hypocretins, which increase feeding. The PVN has receptors for α-MSH, which decreases feeding, and antagonism of these receptors by AgRP increases feeding. The PVN neurons also produce thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin, which can affect energy balance. The DMN is thought to serve as an integrative centre, processing information from other neurons, and, like the VMN, is a target for leptin, which inhibits feeding.

Orexigenic hypothalamic neuropeptides: NPY and AgRP

NPY is a 36 amino acid peptide found in the peripheral nervous system and in the brain. NPY release increases when fasting and decreases about 6 h after feeding. Intra-cerebroventricular administration of NPY increases appetite. NPY production is reduced by α-MSH, leptin, insulin, peptide YY (PYY), glucose and oestrogens and increased by ghrelin, orexins and glucocorticoids. NPY binds to G protein-coupled receptors NPY1R to NPY5R; the Y1 and Y5 receptors are up-regulated during fasting, whereas Y2 and Y4 have autoinhibitory effects on NPY. NPY binding to the Y5 receptor stimulates MCH and orexin-producing cells in the LaH, while binding of NPY to the Y1 receptor suppresses the anorexigenic POMC cells in the ARC. NPY decreases energy expenditure by inhibition of PVN neurons that produce TRH and CRH, thus decreasing brown fat thermogenesis and inhibiting the thyroid axis.

A related peptide, PYY, is localized in endocrine cells of the intestinal mucosa. NPY and PYY are also involved in regulating blood flow, motility and electrolyte secretions in the intestine. Leptin administration decreases feed intake (see Section 3.5) and this occurs at least in part by decreased activity of NPY. Leptin receptors have been co-localized with NPY neurons in the ARC of the hypothalamus. Animals that do not produce functional leptin (such as *ob/ob* mice), or that do not have functional leptin receptors, over-express NPY and are hyperphagic. This has led to the proposal that NPY is the major mediator of the actions of leptin.

AgRP is one of the most potent and long-lasting peptides that stimulate appetite. AgRP exerts its

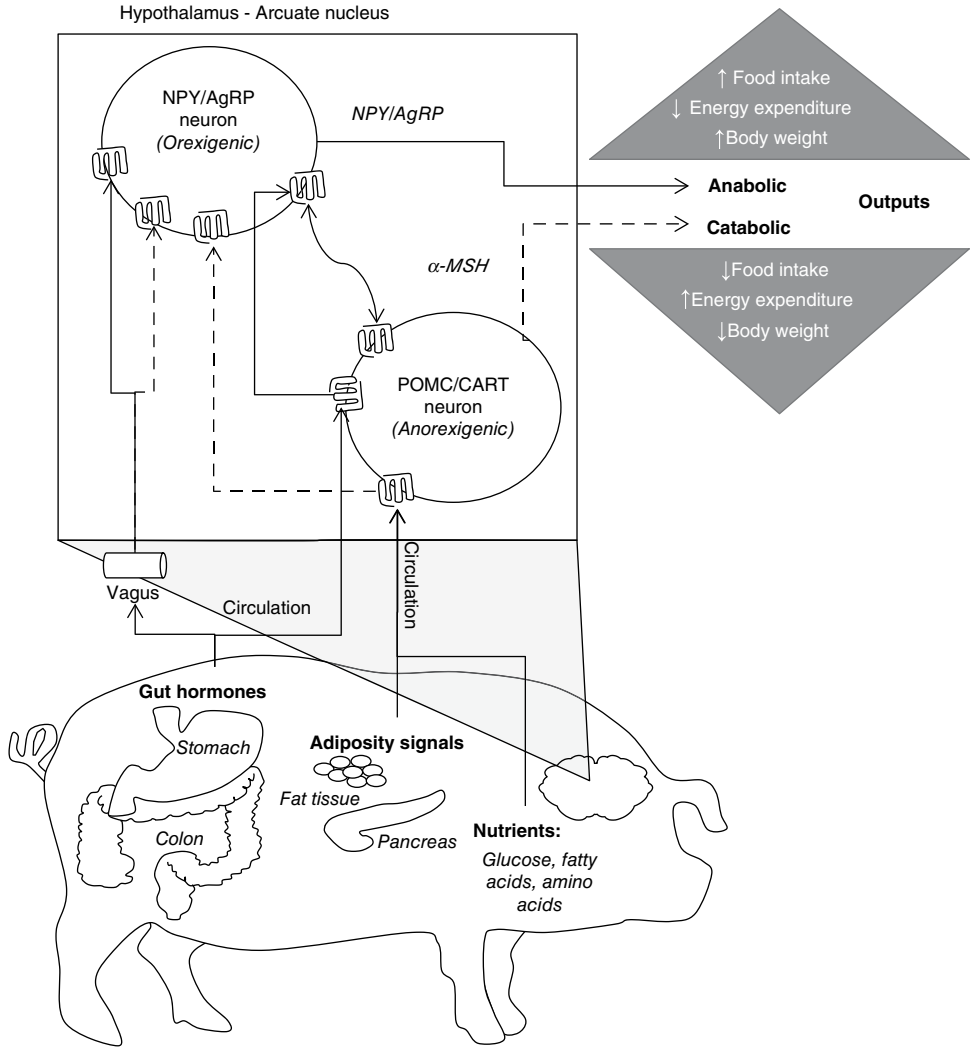


Fig. 3.32. Organization of endocrine signals controlling food intake.

action by inhibiting the melanocortin receptor, which blocks the reduction of food intake. The action of AgRP lasts much longer than NPY, and AgRP may also act in the paraventricular nucleus to activate NPY.

Anorexigenic hypothalamic neuropeptides: POMC/MSH and CART

The POMC molecule is cleaved by prohormone convertases PC1 and PC2 to produce α-MSH, or melanocortin. The levels of circulating POMC reflect energy status by inhibiting feed intake via α-MSH and enhancing energy expenditure. The production of α-MSH in the ARC rather than in the pituitary has an effect on appetite. α-MSH exerts its actions through melanocortin 3 and 4 receptors (MC3R, MC4R) to reduce feed intake. Inhibition of these receptors leads to hyperphagia and obesity. α-MSH binding to MC3R inhibits NPY-producing cells in the medial ARC. NPY and AgRP block the inhibitory effect of α-MSH via MC4R on the orexigenic MCH-producing cells of the LaH to increase feed intake.

CART is co-expressed with α-MSH in the ARC. It is widely expressed in the nervous system, pituitary

endocrine cells, adrenomedullary cells, islet somatostatin cells and in rat antral gastrin cells. CART induces TRH release from the PVN, leading to an increase in energy expenditure. CART levels decrease during fasting and are increased by leptin. CART levels are suppressed in leptin-deficient (*ob/ob* mice) and are restored by leptin administration. CART expression is also modulated by glucocorticoids. CART cells in the nucleus tractus solitarius (NTS) in the brain stem also possess cholecystokinin (CCK) receptors, suggesting that CART may play a role in the satiety effects of CCK. CART is up-regulated by ingestion of cocaine and its derivatives, and cocaine and amphetamines are weight-loss ingredients in many anti-obesity drugs that tend to be illegal in Canada. For more information on hypothalamic neuropeptides, see the review by Yoo *et al.* (2021).

For interest

The loss of function of the MC4R receptor is the most common genetic cause of obesity in humans and occurs in 3–5% of people with extreme obesity.

Downstream pathway: orexin and MCH

Orexin (or hypocretin) is a neuropeptide derived from prepro-orexin in the LaH and has two active forms: orexin-A and orexin-B. Orexin-A is composed of 33 amino acids with a pyroglutamyl residue at the amino terminal, two disulfide bonds and carboxy terminal amidation. Orexin promotes food intake and exerts its action through two GPCRs: orexin-A binds both OX1R and OX2R with high affinity, while orexin-B displays more selectivity for OX2R. The orexin-A peptide has a more potent effect on increasing feeding than orexin-B and mediates gastric secretion via the vagus nerve. Orexins are less potent orexigenic molecules than NPY and their effect is of short duration. MCH also promotes food intake, and neurons expressing MCH and orexin may work cooperatively to increase feed intake. For more information on orexin, see the review by Polito *et al.* (2020).

Gastrointestinal tract hormones

Hormones produced by the gastrointestinal tract (GIT) are secreted into the circulation by specialized enteroendocrine cells in response to specific dietary nutrients and act as 'satiation signals'. They optimize the process of digestion and nutrient absorption and control the rate at which nutrients are delivered to different gut compartments. They also act in a coordinated manner to signal from the GIT to the brain to regulate feed intake. Ghrelin is the only known orexigenic gut hormone, while CCK, the proglucagon-derived peptides GLP-1 and oxyntomodulin (OXM) and the PP-fold peptides PYY and PP are anorexigenic factors produced by the GIT. Most, if not all, of these hormones are also synthesized in the brain, so their effects may not come directly from the circulation. However, their effects on appetite are generally short lived and are not involved in long-term changes in energy balance and body weight. The endocrine changes that occur in response to feeding are illustrated in Fig. 3.33 and the endocrine responses to fasting are shown in Fig. 3.34.

Ghrelin and obestatin

Ghrelin is a 28 amino acid peptide produced in the stomach, kidney and placenta and is the endogenous ligand of the growth hormone secretagogue receptor (GHS-R) (see Section 3.4). It is the most potent known circulating orexigen and has been called the 'hunger hormone'. Signalling by ghrelin is mediated downstream by neurons of the ARC expressing NPY and AgRP, which are two potent orexigenic peptides.

Ghrelin secretion is influenced by nutritional status and is inversely correlated with body weight. The main factors promoting the production of ghrelin include fasting, hypoglycaemia and leptin. The main inhibiting factors of ghrelin secretion are food intake, obesity and hyperglycaemia. Ghrelin acts to initiate feeding activity and decrease energy expenditure by limiting fat catabolism. Ghrelin decreases the latency of feeding and subsequently plasma ghrelin levels decrease following feeding. Ghrelin secretion decreases dramatically following gastric bypass surgery. A 23 amino acid peptide, obestatin, is produced from preproghrelin and, in contrast to ghrelin, it is thought to produce an anorexigenic response and balance the effects of ghrelin. While ghrelin inhibits insulin secretion, obestatin potentiates the insulin response. In addition, while ghrelin stimulates the release of growth hormone, obestatin attenuates this effect. Thus, the ghrelin/obestatin balance could be essential for determining food intake. For more information, see the review by Hassouna *et al.* (2010).

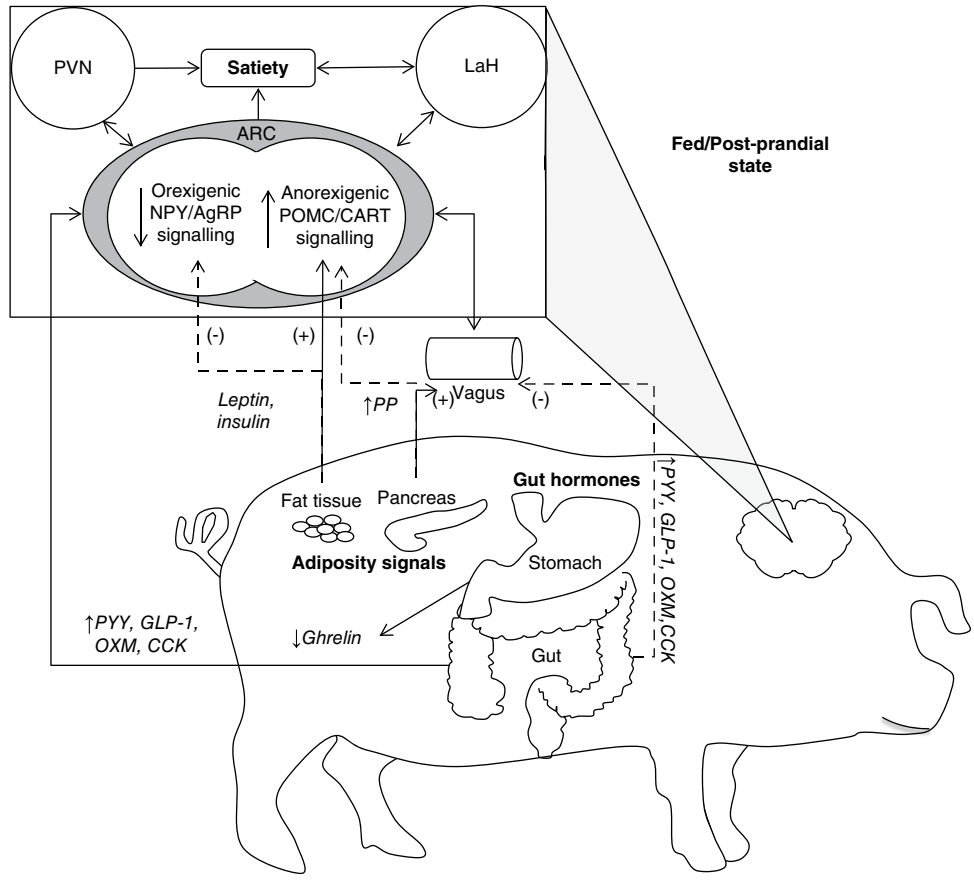


Fig. 3.33. Endocrine changes in the fed/post-prandial state (adapted from Gardiner *et al*., 2008).

Cholecystokinin

Cholecystokinin (CCK), a peptide hormone produced by the duodenal mucosa, was the first GIT hormone to be implicated in the short-term regulation of feed intake. The transfer of digesta (particularly amino acids, HCl and certain fatty acids) from the stomach to the duodenum stimulates its release. CCK is released locally to act in a paracrine manner and also into the circulation in response to nutrients, where it remains elevated for 3–5 h. CCK stimulates emptying of the gall bladder and release of pancreatic enzymes, delays gastric emptying by reducing pylorus contractions and vasodilation and increases motility in the intestine. These effects are mediated via high-affinity CCK-A receptors (CCKAR). CCK works synergistically with secretin for the production of water and bicarbonate by the pancreas. CCK, or a related peptide, also acts as an important satiety signal to reduce food intake by decreasing meal size in a dose-dependent manner. Interfering with CCK function can potentially be used to increase food intake.

CCK is structurally related to gastrin (Fig. 3.35) and the common C-terminal pentapeptide is present in a wide range of species. The tyrosine at residue 7 from the carboxy terminal of CCK is sulfated, and the sulfated form of the hormone binds 160 times more strongly to the GPCR cholecystokinin receptor (CCKAR) in the gastrointestinal tract than does the non-sulfated form. Another form of the receptor (CCKBR), which binds gastrin, is located primarily in the brain. CCK exists in a number of forms and all of them are derived by processing of the 115 amino acid preprocholecystokinin. CCK-58 is the most processed form of CCK and is found in most tissues. The 8 amino acid C-terminal peptide (CCK-8), which is found in the central nervous system, is inhibitory to food

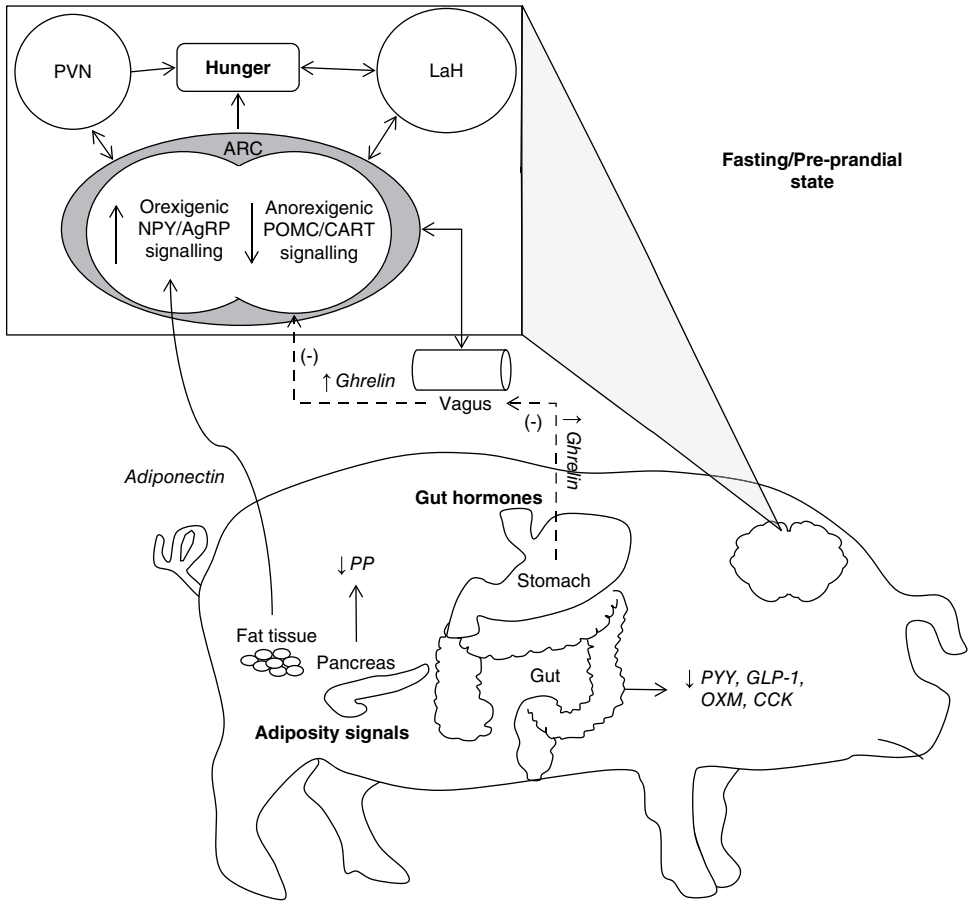


Fig. 3.34. Endocrine changes in the fasting/pre-prandial state (adapted from Gardiner *et al*., 2008).

$$\text{Gastrin}_{(17)}\ \text{(pyro)Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr(SO}_3\text{H)-Gly-Trp-Met-Asp-Phe-NH}_2$$

$$\text{CCK}_{(17\text{-}33)}\ \text{Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO}_3\text{H)-Met-Gly-Trp-Met-Asp-Phe-NH}_2$$

Fig. 3.35. Structures of gastrin and cholecystokinin.

intake as well as stimulating pancreatic secretions and gall-bladder contraction.

Synthetic analogues of CCK inhibit food intake as well as inducing gall-bladder contraction and release of pancreatic enzymes, suggesting that these functions are regulated by a common mechanism. Satiety signals are transferred to the hypothalamus via the vagus nerve but there is evidence that CCK also acts locally within the ventral medial hypothalamus. This is another example, along with somatostatin and many of the hormones described in this section, of a hormone that has actions in both the gut and the brain.

Proglucagon-derived peptides

Proglucagon is processed in a tissue-specific manner to form a number of proglucagon-derived peptides (PGDPs): glucagon in the α-cells of the pancreas and glucagon-like peptides (GLPs) in the enteroendocrine cells of the intestine and in the brain (Fig. 3.36).

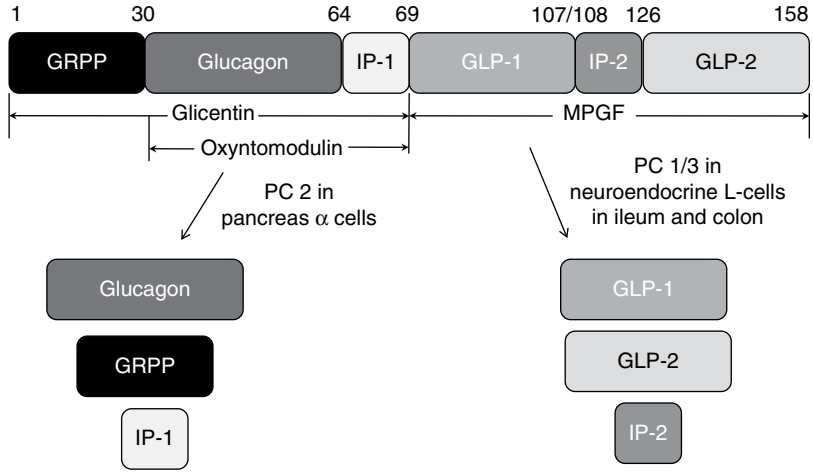


Fig. 3.36. Structural organization and processing of proglucagon and the proglucagon-derived peptides. The numbers refer to the amino acid positions of the individual peptides within proglucagon. GLP, glucagon-like peptide; GRPP, glicentin-related pancreatic peptide; MPGF, major proglucagon-derived fragment; IP, intervening peptide.

The prohormone convertase (PC) enzyme 2 is responsible for the production of glucagon, while cleavage by PC1/3 results in the production of GLP-1 and GLP-2. Glucagon is released in response to decreases in blood glucose (see Section 3.8), while GLP-1 and GLP-2 increase β-cell and intestinal mucosal growth, respectively. Glicentin inhibits gastric acid secretion but does not affect food intake.

GLP-1 and oxyntomodulin (OXM) are released into the circulation by L-cells of the ileum and colon in proportion to the amount of energy ingested and act as satiety signals to reduce food intake. Both GLP-1 and OXM stimulate insulin secretion and are therefore known as incretins. The two forms of GLP-1 synthesized are GLP-1 1-37 and GLP-1 1-36 amide, which are further cleaved to remove the six N-terminal amino acids. GLP-1 1-36 amide is also synthesized within the CNS and inhibits food intake, at least in part by eliciting feelings of stress or malaise. Peripheral GLP-1 signals to the brain via the vagus nerve and is part of the 'ileal brake', as it acts to slow gastric emptying and reduce the transit of food through the GIT. GLP-1 analogues are used for the management of type 2 diabetes mellitus.

OXM slows gastric emptying and inhibits gastric acid secretion. The anorexic effect of OXM can be blocked by the GLP-1 receptor antagonist exendin, which is a 39 amino acid peptide isolated from the venom of the Gila monster, *Helloderma suspectum*. The effects are also absent in GLP-1 receptor knockout mice, so it is likely that OXM acts via the GLP-1 receptor. OXM also increases energy expenditure in addition to decreasing feed intake. For more information, see Lafferty *et al*. (2021).

Peptide YY and pancreatic polypeptide

Peptide YY (peptide tyrosine tyrosine) (PYY) and pancreatic polypeptide (PP) are PP-fold peptides of 36 amino acids that share a common hairpin structural motif that is necessary for receptor binding. They are part of the anorexigenic pathway and also part of the 'ileal brake', since they affect motility of the GIT. PYY has two endogenous forms, PYY1-36 and PYY3-36, the latter produced by cleavage of two N-terminal amino acids by dipeptidyl peptidase IV (DPP-IV). PYY is mainly secreted from the L cells of the distal GIT in proportion to the caloric content of the meal, whereas PP is mainly secreted from the pancreas, colon and rectum. They both exert their action by binding to the NPY G protein-coupled receptors NPY2R and NPY4R. PYY acts primarily via the Y2 receptor, which is highly expressed in the NPY neurons in the ARC, and may reduce feed intake by inhibiting NPY release or increasing POMC release. PYY levels rise rapidly after eating, plateau after 1–2 h and remain elevated up to 6 h after eating. PP acts via the Y4 receptor and is released in proportion to the number of calories ingested, remaining elevated for 6 h after eating.

Peripheral signals of adiposity

Adiposity signals are related to the size of body fat stores and include leptin and insulin. Leptin is secreted by adipose tissue and acts via the hypothalamus to affect food intake and energy utilization. The production of leptin (see Section 3.5) is correlated with adipose tissue mass. Leptin functions through a JAK-STAT-type receptor (LepR) (see Section 1.3) and regulates appetite, whole-body energy balance and body composition. Circulating leptin crosses the blood–brain barrier and binds to the LepRb receptor in the ARC, VMN, DMN and LaH. In the ARC, it directly activates anorexigenic POMC neurons and inhibits orexigenic AgRP/NPY neurons to decrease feed intake.

Insulin produced by the β-cells of the pancreas stimulates the uptake of glucose (see Section 3.8). Levels of insulin rise in response to increases in blood glucose, and circulating levels are proportional to fat mass. Insulin also crosses the blood–brain barrier via receptor-mediated transport and has an anorexigenic effect. The anorexigenic effects of both leptin and insulin involve signalling via the melanocortin receptors.

The metabolite *N*-lactoyl-phenylalanine (Lac-Phe) is generated during intense exercise and acts to suppress feeding and obesity. The biosynthesis of Lac-Phe from lactate and phenylalanine is catalysed by the cytosol non-specific dipeptidase (CNDP2) protein, which is expressed in macrophages, monocytes and other immune and epithelial cells in different organs. Chronic administration of Lac-Phe decreases adiposity and body weight and improves glucose homeostasis. This may be the molecular mechanism that explains the metabolic benefits of exercise. For more information, see Li *et al*. (2022).

Applications of dietary control

Weaning weight in pigs was positively correlated with the expression of NPY, AgRP, orexin and orexin receptor (OX2R) genes in the hypothalamus, which highlights the individual predispositions that regulate how an animal eats and grows. Differences in plasma levels of CCK have also been found in pigs with different genetic potential for feed intake, and the satiety effects of CCK are one of the genetic differences between lines of pigs for feed intake. Decreased production of CCK has been found in humans with bulimia nervosa and increased levels of CCK or sensitivity to CCK have been implicated in age-related anorexia. CCK may prove to be a useful target in the control of obesity in humans.

Immunizing pigs, but not sheep, against CCK-8 increased growth rate by about 10% and feed intake by 8%, with no change in carcass composition. Antibody titres were correlated with weight gain. Treatment of pigs and immature rainbow trout with a cholecystokinin A receptor (CCKAR) antagonist (Devazepide, MK-329) (Fig. 3.37) increased feeding behaviour, feed intake and weight gain. Active immunization against ghrelin also results in a decrease in feed intake and slower growth.

For interest

Two SNPs in the CCKAR gene promoter have been associated with increased body fatness. Polymorphisms in the Y2 receptor and in the PYY gene are associated with increased obesity.

The focus of control of feed intake in humans is usually on decreasing feed intake to reduce obesity. However, in some disease states that cause appetite loss and wasting, it is necessary to promote appetite. Ghrelin infusions increase feed intake, and orally active ghrelin agonists have been developed. Endocannabinoids also promote appetite and the consumption of cannabis promotes hyperphagia. This is commonly used medicinally for patients with diseases such as AIDS or certain cancers. The lipophilic tetrahydrocannabinol (THC) factor has GPCRs CB1 and CB2 located predominantly on the CNS. CB1 knockout mice were leaner and did not exhibit hyperphagia. Injection of the opioid molecule syndyphalin (SD-33) before weaning increased feed intake in pigs from 9 days after weaning. Novel

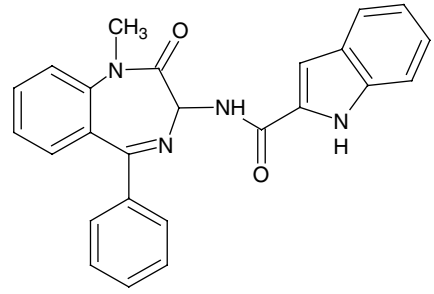


Fig. 3.37. Structure of a CCKAR antagonist (MK-329).

opioid therapies may be used to improve well-being in animals during periods of stress such as weaning, castration, or transport.

Analogues of PYY are being investigated for clinical use in the treatment of obesity, since the anorexic effect of PYY is preserved in obese individuals, which are resistant to leptin. Novel non-peptide analogues have been developed that are specific for the Y1, Y2, Y4 and Y5 forms of the PP-fold peptide receptors. These have been useful tools in deciphering the physiological roles of these different receptor isoforms. The gut hormones are degraded and inactivated by the enzyme DPP-IV, and the half-life of GLP-1 is only 2 min. Longer-acting GLP-1 receptor agonists have been developed for the treatment of diabetes and obesity in humans, such as exenatide, lixisenatide, liraglutide, albiglutide, dulaglutide and semaglutide. DPP-IV inhibitors such as sitagliptin and vildagliptin are also being studied.

Although many of the major signalling pathways that regulate feed intake are shared between mammals and birds, there are some differences in the function of specific peptide signals. PYY and PP are anorexigenic in mammals but orexigenic in birds. While ghrelin and GHRH stimulate feed intake in mammals, they are anorexigenic in birds. MCH, orexins, galanin and motlin are orexigenic in mammals but have no effect in chickens. For more information on feed intake in poultry and pigs, see Everaert *et al*. (2019).

3.11 The Gut Microbiome and Antimicrobials

Key concepts

- The gut microbiome is a diverse microbial community that produces nutrients and signalling molecules to interact with the host animal.
- The microbiome acts as an endocrine organ to affect the overall health and performance of the host animal.
- Antimicrobial growth promoters (AGPs) have been used in livestock feeds to improve growth and feed efficiency by altering the gut microbiome.
- Some countries have limited use of AGPs because of concerns about the development of antimicrobial-resistant strains of bacteria.
- Prebiotics and probiotics can alter the microbiome to enhance the growth/activity of specific beneficial microbes and improve animal productivity and health.

The gut microbiome

The gut microbiome is a complex microbial community that coexists inside the intestinal tract of the animal. It is composed of different types of microorganisms; over 500 species of bacteria, as well as archaea, fungi, viruses and phages, colonize the intestine. The number of bacteria in the intestine is comparable to the number of cells in the animal body and the microbiome contains 7.5 million bacterial genes, which is about 38 times more genes than in the human genome. This suggests that the metabolic capacity of the gut microbiota can exceed and complement the host animal's metabolic capacity.

The microbiota interacts with the host in the exchange of nutrients and metabolites, influencing immune function and inflammation, control of pathogens and the function of the digestive system. The microbiota provides fermentation products to the animal and prevents the colonization of pathogens by competitive exclusion, but it can also compete with the host for other nutrients. Different metabolites and nutrients produced by the microbiota have a substantial influence on the metabolism, physiology and health of the host. The microbiome can thus be considered an endocrine organ within the animal, where bacterial metabolites act as paracrine or endocrine factors to regulate host metabolism. Conversely, the host animal's genetics, nutrition, physiology and disease status can affect the composition and function of the microbiome (Fig. 3.38).

Effects on animal production and health

Initial studies on the gut microbiota were focused on its association with infectious diseases of the gut (e.g. *Escherichia coli, Shigella*), along with acute colitis, Crohn's disease and inflammatory bowel disease. Treatment of these diseases with antibiotics can eliminate beneficial microbes along with the target pathogens to cause dysbiosis, a condition where there is an imbalance between the host animal and the microbiome. Widespread use of antibiotics in livestock and aquaculture is also raising safety concerns about increasing antibiotic resistance in bacteria. Modifying the composition of the gut microbiota can impact the metabolism of the

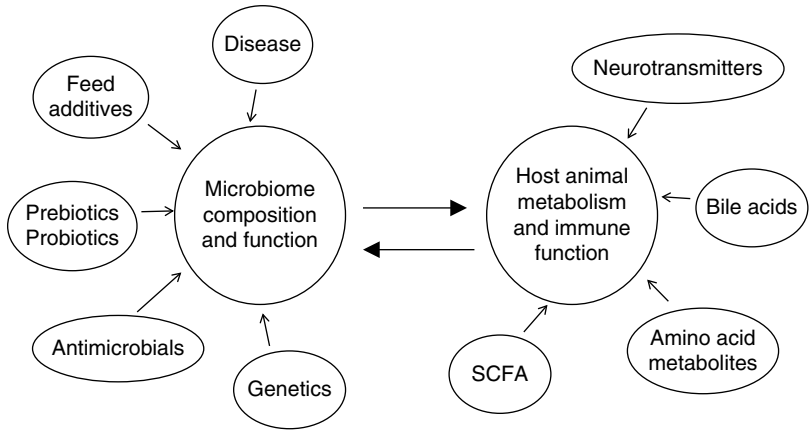


Fig. 3.38. Interactions between the gut microbiome and host animal.

animal. For example, germ-free mice extract less energy from food, demonstrating that bacteria can affect intestinal energy extraction and homeostasis by influencing the digestion and intestinal absorption of dietary components.

Some constituents of bacteria can trigger the onset of low-grade inflammation, and the barrier function of the gut must be able to prevent these immunostimulatory molecules (lipopolysaccharides (LPS) and other pathogen-associated molecular patterns (PAMPs) such as flagellin and peptidoglycans) from entering the circulation from the intestine. At the same time, the gut barrier must also allow uptake of essential nutrients and fluids from the intestine. Alteration of intestinal homeostasis by altering the composition of the gut microbiota or gut barrier function can also alter the secretion of gut endocrine hormones, thereby altering metabolism.

Bacterial metabolites act as paracrine or endocrine factors

In addition to microbial components such as LPS, several microbial metabolites are involved in the communication between the host and the gut microbiome. This includes short-chain fatty acids (SCFAs) produced from fermentation of dietary fibres, secondary bile acids, and metabolites produced from some amino acids. Their physiological effects include the regulation of energy, glucose and lipid metabolism and the modulation of inflammation and immune response.

Fermentable dietary fibres, such as inulin-type fructans that include inulin and oligofructose, are not digested in the upper GI tract and are metabolized to SCFAs acetate, butyrate and propionate by specific bacteria in the intestine. The chemical structure of the fermentable fibres affects the quantity of butyrate, acetate, or propionate produced; inulin is described as propionogenic, whereas resistant starches are more butyrogenic. SCFAs are an important energy source for the host, but they also bind to and activate the G protein-coupled receptors GPR-43 (also known as free fatty acid receptor 2 (FFAR2)) and GPR-41 (also referred to as FFAR3) that are present on the intestinal enteroendocrine L cells. Activating these receptors promotes the secretion of gut peptides, GLP-1, PYY and proglucagon, to regulate feed intake, blood glucose levels and insulin sensitivity (see Section 3.10). SCFAs can also enter the circulation and be transported to other organs such as liver, adipose tissue, brain and muscle to cause systemic effects. SCFAs can also be sensed by the gut bacteria themselves and contribute to bacterial cell–cell interactions by 'quorum sensing' to regulate processes such as motility, biofilm formation and colonization by pathogens.

The gut microbiota also affects both bile acid synthesis and the production of secondary bile acids. Bile acids bind to the membrane receptors TGR5 (Takeda G protein-coupled receptor 5) or Gpbar1 (G protein-coupled bile acid receptor 1) expressed on the L cells and improve liver metabolism and glucose tolerance by regulating intestinal GLP-1 production. In the adipose tissue, the TGR5 receptor induces expression of the 2-deiodinase (DIO2) enzyme, which activates thyroid hormone

T_4 to the active form T_3. Bile acids also activate the farnesoid X receptor (FXR), which regulates glucose tolerance and insulin sensitivity.

Some compounds derived from bacterial metabolism of amino acids can negatively affect host metabolism. For example, branched-chain amino acids produced by bacteria are linked to insulin resistance. Imidazole propionate (ImP), a bacterial metabolite derived from histidine, also contributes to insulin resistance by activating the p38g/p62/mTORC1 pathway and subsequently inhibiting insulin receptor substrate activity. Other bacterial metabolites such as indole and its derivatives (indole-3 propionic acid (IPA), indole-3-acetaldehyde, indole-acrylic acid, indole-3 aldehyde and indole-3 acetate), produced from tryptophan metabolism, have beneficial effects on host metabolism. IPA binds to pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) to reinforce gut barrier function, increase immune response and exert anti-inflammatory effects.

Gut microbes can also influence host metabolism by synthesizing classical neurotransmitters (histamine, serotonin, GABA, catecholamines) or gaseous neurotransmitters (NO and H_2S). Those neurotransmitters have a local impact on gut motility and intestinal hormone release and can impact cognition and behaviour via the link between the enteric nervous system and the brain. Gut microbes also affect the endocannabinoid system by producing *N*-acyl amide, an endocannabinoid mimetic that influences host glucose metabolism via the GPR119 receptor. The endogenous ligands of GPR119 are oleoyl ethanolamide and 2-oleoyl glycerol. Activating GPR119 on the enteroendocrine L cells stimulates GLP-1 secretion to regulate glucose and energy metabolism. Specific proteins constitutively expressed by bacteria (caseinolytic protease B (ClpB), Amuc_1100) can act as endocrine factors and affect host metabolism via a paracrine or endocrine action. ClpB has sequence homology with melanocyte-stimulating hormone (MSH) and thus is involved in regulation of appetite (see Section 3.10). Amuc_1100, expressed on the outer membrane of probiotic bacteria, improves barrier function by activation of Toll-like receptor 2 in the innate immune system.

Evaluation of the composition of the microbiome

A number of methods have been used to evaluate the composition of the microbiome. These include taxonomic profiling using 16S rRNA sequencing, whole metagenomic sequencing analysis, estimation of the genes expressed by the gut microbes (gene count) and quantifying the absolute number of bacteria (microbial load or cell counts). There is no single gold-standard method to analyse the gut microbiome; the best strategy is using a combination of these approaches, potentially along with metabolomics to measure the different metabolites produced by the microbiota.

Microbial diversity can be determined via two different sequencing approaches: amplicon sequencing and shotgun metagenomics. In the amplicon sequencing method, specific regions of the microbial DNA are amplified (i.e. 16S rRNA gene) and sequenced. In this approach, DNA is extracted and a target variable region of the small ribosomal subunit RNA gene is amplified and sequenced. This method relies on the fact that the 16S rRNA gene is conserved among bacteria but contains a hypervariable region that can be used to identify different bacteria taxa. The shotgun metagenomics approach does not target a specific gene but sequences the whole microbial genome of the sample. After sequencing, the individual reads are assembled together and aligned to a reference genome, allowing for the identification of large numbers of coding and non-coding sequences. This information can be used for functional analyses to construct metabolic pathways based on enzyme coding genes. However, this method is costly, which limits its application for routine collection and selection purposes.

Given the profound effects that the microbiome has on host metabolism, a major challenge is determining the function of different components of the microbiome (bacterial communities, strains and metabolites) so that an ideal microbiome can be designed to optimize animal productivity and health. At the taxonomic level, 90% of gut bacteria in monogastrics belong to the phyla *Firmicutes, Bacteroidetes* and *Actinobacteria*, with the remaining portion belonging to *Proteobacteria* and *Verrucomicrobia*. The diversity of bacterial species and their abundance in the gut increases with animal age, as the growing animal is exposed to an orderly succession of bacterial populations, with the end result being a stable, diverse microbiota population. The genetics of the host animal also affects the composition of the gut microbiome, which can differ dramatically among individual animals. Once a stable microbiome has been established

in the adult animal, the composition of the microbiome tends to return to this stable condition following short-term changes imposed on it.

The composition of the gut microbiota is altered in diseases such as type 2 diabetes, with a lower relative abundance of *Firmicutes clostridia*, which have anti-inflammatory properties, but higher Betaproteobacteria in diabetic humans. *Faecalibacterium prausnitzii* is associated with improvements in glucose tolerance. Branched-chain amino acids produced by *Bacteroides vulgatus* species are linked to insulin resistance. The glucose-lowering drug metformin can increase SCFA-producing bacteria and mucin-degrading *Akkermansia muciniphila* while increasing intestinal glucose absorption and GLP-1 production. For more information, see Rastelli *et al*. (2019) and Régnier *et al*. (2021).

Factors affecting the composition and function of the microbiome

Antimicrobial growth promoters

The prophylactic use of antimicrobial growth promoters (AGPs) for enhancing meat production began in the 1940s, when it was discovered that they improved growth rate and feed conversion efficiency when used in small amounts in the diet. This amounts to 4–20% improvement in growth rate and 4–10% improvement in feed efficiency of pigs and poultry. These effects may be greater in younger than in older animals. There are no significant effects on carcass quality, such as length and depth of the carcass, fat content and dressing percentage.

AGPs may improve growth and feed efficiency by altering the gut microbiome, since the effects are not seen in germ-free animals and many of these AGPs are not absorbed from the gut. The performance benefits could be caused by the suppression of bacteria that under normal conditions would cause malabsorption of nutrients such as fat, protein, carbohydrates, vitamins and minerals. They may reduce the production of toxins, prevent the growth of pathogens, decrease the destruction of nutrients and increase the structural integrity and absorptive capacity of the intestinal mucosa. There may also be direct effects of antibiotics on the metabolism of the animal. Antibiotics may reduce low-level and sub-chronic infections and thus cause increased growth performance. This is supported by experiments in which animals treated with bacterial LPS or immune mediators such as IL-1 (see Section 6.3) have reduced efficiency, consistent with an animal that has not been treated with AGPs.

In ruminants, antibiotics can affect rumen fermentation and improve digestibility of feeds. Many of these compounds are ionophores, which are also effective coccidiostats in poultry. Improvements in daily gain of 15–25% and feed conversion efficiency of 3–16% are seen with beef cattle fed these antibiotics. The extent of the effects is dependent on diet, with greater improvements in grazing animals eating low-quality forages compared with those fed high-energy-concentrate diets.

A number of factors can affect the economic advantages of using AGPs. Responses in feed conversion and growth rate obtained in controlled experiments may not be obtained under field conditions. One exception would be the use of in-feed AGPs, which can give greater responses in commercial use, due to the higher bacterial challenge and feed variability that are likely under production conditions. The economic benefits can also be affected by the duration of treatment that is necessary. Changes in health status, either positive or negative, may result in effects that continue long after treatment is completed. Changes in the volume and composition of manure may be important in regions that have strict environmental controls, such as the Netherlands. Increasing the lean content of carcasses makes economic sense only if the premiums offered to the producers for lean are greater than the cost of producing the extra lean. In some situations, such as for fresh pork cuts, a low fat content is desirable, while for some processed products a higher fat content is desirable.

The World Health Organization (WHO) has recommended that better animal health management practices be adopted so that the prophylactic use of antimicrobials can be eliminated. A link between the use of AGPs in animal feeds and the development of antimicrobial-resistant (AMR) strains of bacteria was suggested as early as the 1950s. Antimicrobial resistance genes can be passed from animal to human microbiota, and pathogenic bacteria resistant to a number of antimicrobial agents appeared worldwide in the 1980s. The potential carry-over of antibiotic residues to humans is also a concern. This has led to the banning of some feed-delivered antibiotics. Of particular concern is the use of antimicrobials that are in the classes that are medicinally important for humans. The WHO has recommended that all

countries monitor the use of antimicrobial drugs in animals so that this can be compared with the incidence of AMR bacteria. Recommendations to reduce or eliminate the use of antimicrobials in feed were made as early as 1969, and in 1986 Sweden was the first country to eliminate the use of antimicrobials for growth promotion. Avoparcin was banned in Denmark in 1995 and by the entire EU in 1997, in response to concerns about vancomycin-resistant enterococci (VRE) in animals. In 1999, there was an EU-wide ban on the use of most AGPs in feeds, with a complete ban enforced in 2006. The use of medicinally important antibiotics for growth promotion was removed in the USA in 2013 and fully implemented by 2017. The use of antimicrobials is now restricted to therapeutic use, by prescription only. For more information on antimicrobial resistance, see Ferri *et al*. (2017) and Muurinen *et al*. (2021).

Alternative feed additives to AGPs

Because of the problems associated with the use of AGPs, a number of different alternative feed additives that promote feed utilization and growth have been investigated. Adding enzymes to feeds to increase their digestibility and decrease the viscosity of the digesta can alter the gut microflora. Feeding organic acids can make adjustments to the microbiome to increase enzyme activity and enhance amino acid digestibility, as well as reduce upper gut pH to reduce colonization by acid-intolerant bacteria. Phytobiotics are plant-derived products, including a wide variety of herbs, spices and essential oils, which can be used as feed additives to improve performance. These natural products are complex mixtures of bioactive components that have synergistic antimicrobial and immune-enhancement activities to improve gut functions. Some products also have antioxidant properties to improve the oxidative stability of animal products. However, the content of active substances can sometimes vary widely, depending on the plant part used, harvesting season and geographical origin. As such, efficacy, toxicity and safety assessments are needed before they can be used extensively. For more information on alternatives to AGPs, see Stoica and Cox (2021).

Dietary fibre, fat, protein and carbohydrates are key dietary factors that are processed to varying degrees by both the host and gut microbes and they impact how microbes affect host metabolism. Micronutrient- and microbiota-derived factors may also participate in host–microbe responses. Excess intake of dietary fat alters the composition of the intestinal microbiota to cause dysbiosis, resulting in proinflammatory responses in the host. Digestible carbohydrates are easily processed by the host for energy, whereas indigestible carbohydrates can be processed by gut bacteria.

Due to the influence of the microbiome on overall health and performance, many researchers have focused on manipulating or maintaining the stability of the microbiome, so that harmful and pathogenic bacteria are replaced by beneficial ones. Methods to alter the microbiome include supplementation to enhance the growth/activity of specific beneficial microbes, and selective targeting to alter a specific member of the microbiome, rather than altering the microbiome as a whole. Supplementation involves the addition of beneficial cultured strains into the microbiome community (probiotics), the addition of nutrients that promote the growth of beneficial microbes (prebiotics), or a combination of a probiotic and a prebiotic (called a synbiotic), which can sometimes be used for a synergistc effect. A popular synbiotic is a combination of *Bifidobacterium* or *Lactobacillus* genus bacteria with fructo-oligosaccharides (FOSs).

Prebiotics are non-digestible feed ingredients that stimulate the growth of endogenous beneficial bacteria at the expense of pathogenic bacteria. The addition of a prebiotic favours a balanced microbiome which then leads to good performance. Prebiotics include various types of fermentable carbohydrates; the prebiotics most commonly used in livestock nutrition are FOSs, galacto-oligosaccharides (GOSs), inulin, isomalto-oligosaccharides (IMOs), xylo-oligosaccharides (XOS), lacticol, lactulose and cereal fibre. A number of prebiotic formulas intended for livestock are commercially available. The true benefits of feeding these supplements may only be seen when animals are reared under commercial conditions where a pathogenic microbial challenge is present. While optimum rates of supplementation can promote growth, higher inclusion rates can sometimes decrease growth or cause digestive disorders such as diarrhoea.

Probiotics or direct-fed microbials (DFMs) are live strains of strictly selected microorganisms which confer a health benefit on the host. Most often they are Gram-positive bacteria such as *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Streptococcus*, but they also include some

fungi and yeast strains of *Saccharomyces cerevisiae* and *Kluyveromyces* species. Probiotics can compete with pathogens for nutrients and prevent colonization via competitive exclusion, obstructing attachment sites and producing metabolites such as SCFAs, to maintain balance within the microbiome. Gut bacteria can regulate intestinal motility by affecting the intestinal nervous system and improve gut barrier function. Probiotic microbes can also improve host immunity against pathogenic microorganisms by altering biofilms, epithelial cell turnover and producing antimicrobial molecules and cytokines. Probiotics can enhance the function of the permeability barrier of the intestinal mucosa through phosphorylation of cytoskeletal and tight junction proteins. Lactic acid bacteria produce antimicrobial compounds known as bacteriocins, which allows them to outcompete other bacteria and prevent their colonization. Sphingolipids produced by the dominant commensal *Flectobacillus major* contribute to the mucosal immune system of rainbow trout. Certain probiotics, such as live yeast, can help to stabilize gut pH and decrease the risk of acidosis, a common condition affecting ruminants. The different needs across developmental stages should also be considered in probiotic use. For example, a calf may require microbes able to utilize fibre whereas adult cows will also need microbes able to utilize starch to allow for fast growth and production. Probiotics can increase absorption and utilization of feed and increase body weight of various animals, including turkeys, chicken, piglets, sheep, goats, cattle and horses. For more information, see Markowiak and Śliżewska, (2018).

Faecal microbiota transplants (FMT) from a healthy donor can be used to introduce a new population of microbiota to treat dysbiosis in a recipient. For example, this has been used in people whose microbiomes have become dysbiotic with *Clostridium difficile* overgrowth. Fluid from the rumen of fistulated healthy cows is frequently transplanted into cows that are recovering from intestinal disruptions and abomasal displacements. However, the efficacy of FMTs is limited and variable, since the microbiomes of healthy individuals are highly host-specific and rapidly re-establish following transplantation of microbiomes from another individual.

Selective targeting of the microbiome using methods such as phage therapy and gene editing can be used to eliminate target organisms without altering the rest of the microbial community. Phage therapy uses a specific phage that targets an individual species or strain of bacteria and it can be as effective as antibiotics in treating certain infections in livestock. Species-specific antimicrobial peptides have also been developed that are capable of precisely targeting pathogenic bacteria. Gene editing via CRISPR/Cas can target a specific gene in a specific strain, to cause self-targeted destruction of the genome or deletion of genomic regions that encode for virulence factors. Edited bacteria can also act as gene transfer agents to confer beneficial functions, or to transfer proteins into host cells. The efficacy of these methods may depend on the animal's developmental stage, and may be more effective early in life when both the microbiome and immune system are changing rapidly. For more information, see Song *et al.* (2019).

Practical applications

The use of probiotics and prebiotics in poultry, pigs and cattle to improve the microbiome has resulted in higher productivity, greater feed efficiency and increased weight gain (Fig. 3.39). In addition, the microbiome in cattle can be manipulated to decrease methane production. In pigs, decreasing post-weaning diarrhoea can be controlled by stabilizing the microbiome. In poultry, beneficial components of the microbiome can improve growth and feed efficiency, and reduce intestinal colonization by pathogenic bacteria and contamination of eggs with *Salmonella*.

Control of methane production in ruminants

Ruminant animals have a specialized digestive system that includes a foregut fermentation chamber: the rumen. It contains a diverse microbial population of anaerobic bacteria, fungi, methanogenic archaea, ciliate protozoa and viruses which uses anaerobic fermentation to convert host-indigestible plant material to nutrients for the animal. The primary products of enteric fermentation of fibres composed of cellulose and hemicellulose are SCFAs (also known as volatile fatty acids (VFAs)), including acetate, butyrate and propionate that are the major substrates for energy production, gluconeogenesis and lipogenesis in ruminants and thus greatly affect animal performance. Methane and carbon dioxide are also produced by the rumen microbiota. Methane is a potent greenhouse gas

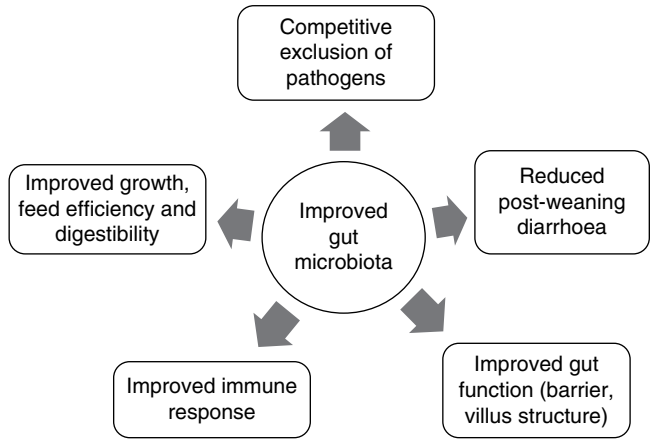


Fig. 3.39. Effects of improved gut microbiome on the host animal.

and has a much higher global warming potential than that of carbon dioxide. Cattle, sheep and goats account for a significant proportion of the methane released into the earth's atmosphere. In addition to environmental concerns, the production of methane decreases the amount of dietary energy that is captured as VFA by 2–12% and thus decreases production efficiency. The composition of the rumen microbiota is different between efficient and inefficient cattle, demonstrating that the composition of the rumen microbiota can affect methane production and feed efficiency of ruminants. The types of VFA produced in the rumen can also be affected by the composition of the feed. Non-structural carbohydrates, such as starch and sugar, are rapidly fermented to decrease the pH and reduce methanogenic bacterial populations, thereby increasing propionate production. Structural carbohydrates such as neutral detergent fibre present in forages are linked to increased methane production.

The negative environmental effects of methane production have led to demands for major reductions in the consumption of red meat to reduce the number of ruminants and amount of methane they release. However, it is projected that demand for red meat will continue to increase at about 1.5% per year to meet the growing population and rising living standards in developing countries. An alternative to reducing red meat consumption or ruminant numbers is to reduce methane emissions from ruminants.

A number of methods for reducing methane production by ruminants have been developed. Two feed supplements – a commercial product, 3-nitrooxypropanol (3-NOP, Bovaer®), and the red seaweed, *Asparagopsis* – can reduce methane emissions by 40% and 90%, respectively. This also increases animal productivity with no adverse effects on health or product quality. Other methods to reduce methane production include genetic selection, vaccination against methanogenic rumen archaea and using other feed additives to alter the gut microbiota.

Anti-methanogenic feed additives can either modify the composition of the rumen microbiome or directly inhibit methanogenesis. The compound 3-NOP inhibits methanogenesis by binding to the active site of the methyl-coenzyme M reductase enzyme, which catalyses the final step in the synthesis of methane by rumen archaea. An effective dose of 3-NOP is around 2% of the diet and is best provided in total mixed rations to feedlot cattle and dairy cows. The red seaweed *Asparagopsis* contains halogenated methane analogues, such as bromoform and dibromochloromethane, within specialized gland cells that it uses as a natural defence mechanism. These compounds competitively inhibit the methyl transfer reactions that are necessary in methane biosynthesis. There are five competing biochemical pathways in the rumen, which produce different amounts of methane and volatile fatty acids, and each of these fermentation pathways is present in specific types of microbes. The anti-methanogenic compounds dibromochloromethane and 3-NOP alter microbial populations to favour those that produce lower amounts of methane.

There is genetic variation in methane emissions among individual ruminant animals and host

genetics may affect the composition of the rumen microbiota. Heritability for methane emission, expressed as methane per unit of feed intake, is moderate (0.13–0.35) in sheep and dairy cattle. The reduction in methane emissions in selected animals can be due to smaller rumen volumes, increased outflow rate of digesta and reduced fermentation in the rumen. This can amount to 3–20% over 10 years, depending on selection pressure. Vaccination against methanogenic rumen archaea has potential to reduce methane emissions in sheep and cattle. However, for a vaccine to be successful, high concentrations of anti-methanogenic archaea antibodies must be transferred from blood to saliva, which is difficult to achieve. Feeding grape marc, nitrate or biochar can affect the rumen microbiota to reduce methane emissions by 10% or less.

It is difficult to permanently modify the established microbiota in mature animals, as the composition of the microbiota tends to revert to its original state following the cessation of treatment. However, modifying the microbiota in the first 3 weeks of life before the microbiota becomes fully established can be more effective in imprinting a desirable and persistent microbiome in the rumen. Changes in the rumen microbial population to reduce methane production can potentially be maintained if treated herds are isolated from non-treated animals. Whole herds with desired rumen populations can potentially be created and maintained through generations provided that they are isolated from animals with different rumen populations. For more information, see O'Hara *et al.* (2020).

Control of post-weaning diarrhoea in pigs

Diarrhoea infections caused by enterotoxic *Escherichia coli* strains are one of the major health problems in pigs in the post-weaning period, resulting in significant economic losses from increased mortality, decreased growth rate and increased veterinary costs. Initially, the digestive system of piglets is colonized by facultative aerobic or anaerobic bacteria. After birth, the gastrointestinal tract is mainly colonized by *Clostridium* and *Enterobacterium* and this is then replaced by bacteria related to the colostrum and milk, mainly *Lactobacillus* and *Bifidobacterium*. This 'milk-oriented microbiome' of nursing animals is influenced by the glycans contained in milk. Weaning is a period of significant changes in the diet of piglets that can cause changes to the gut microbiota. During this period, the chemical, mechanical and immunological barriers of the intestines of piglets are disrupted, which can lead to digestive disorders, diarrhoea and growth retardation, as well as an increased mortality rate.

Post-weaning diarrhoea is a common problem with piglets that is characterized by a decrease in the number of commensal bacteria, including *Lactobacillus sobrius*, *L. acidophilus* and *L. reuteri*, and an increase in pathogenic *E. coli*. This imbalance in the microbiota damages the intestinal villi and increases the permeability of fluids to the intestine, so that piglets are not able to digest carbohydrates that pass into the large intestine, resulting in diarrhoea. For many years, post-weaning diarrhoea has been treated with antibiotics but it can be treated with probiotics and prebiotics. Supplementation with *S. cerevisiae*, *Bacillus licheniformis* or lactic acid bacteria increased the relative abundance of *Lactobacillus* or *Bifidobacterium*, decreased *E. coli* and enhanced production of SCFAs in the gut of weaning piglets. This decreases the pH of the gastrointestinal tract and enhances gut barrier function by providing energy to intestinal epithelial cells. The increase in favourable bacteria increases competition with pathogens for nutrients, decreases their adhesion to intestinal epithelial receptors and increases production of antimicrobial and toxin-inactivating substances. This stabilizes the intestinal microbiota, increases the growth of intestinal villi, improves the digestibility of feed and reduces the incidence of diarrhoea. After the weaning period, there are changes in the gut microbiota due to the influence of diet and environmental factors, which is stabilized after 3 months at the age of about 120 days.

Use in poultry

The use of AGPs in poultry production results in an average 3–5% increase in growth rate and feed efficiency and improved health, but consumer demand for chemical- and antibiotic-free products has led to decreased prophylactic use of antibiotics. Prebiotics and probiotics can replace AGPs and exert similar beneficial effects on poultry. Prebiotics and probiotics act via several mechanisms, including competitive exclusion and antagonism of pathogens, modulation of intestinal microbiota, production of antimicrobial substances, stimulation of the immune system and enhancement of nutrient digestibility and intestinal morphology development.

Poultry probiotics include a number of different microbial strains, including *Lactobacillus*,

Enterococcus, Bifidobacterium, Lactococcus, Pediococcus, Streptococcus, Leuconostoc, Weissella, Bacillus, Saccharomyces and *Candida*. Use of probiotics in broilers significantly enhanced growth rate, FCR and feed intake and improved morphological features of the gut, especially crypt depth and villus height. Probiotics improve intestinal microbial balance to inhibit pathogens through competition for nutrients and receptor sites on intestinal mucosa, as well as the secretion of antimicrobial substances. The stimulation of immunity against invading pathogens is considered to be the most beneficial impact of probiotics supplementation in poultry. Probiotics can protect chickens against the pathogens *E. coli, Salmonella, Campylobacter, Clostridium* and *Eimeria* and thus prevent diseases such as salmonellosis, campylobacteriosis or coccidiosis. Supplementation of birds with the probiotic *Bacillus coagulans* significantly increased the water-holding capacity (reduced drip loss) and tenderness of poultry meat as well as meat yield. Probiotics contribute to increased production and improved quality of eggs and to reduced Salmonella contamination in eggs.

Prebiotics used in the poultry industry include non-digestible oligosaccharides such as mannanoligosaccharides, fructo-oligosaccharides and inulin, galacto-oligosaccharides, xylo-oligosaccharides and isomalto-oligosaccharide and non-starch polysaccharides, such as β-glucan. Prebiotics improve poultry performance and growth by production of SCFAs, mainly butyrate, acetate and propionate, which are absorbed within the GIT and are a major source of energy. Prebiotics can improve the health of chickens by increasing the amount of lactic acid bacteria, including *Bifidobacterium* and *Lactobacillus* and some strains of butyrate-producing bacteria, while simultaneously inhibiting intestinal colonization by pathogenic bacteria, including *E. coli, Clostridium perfringens, Salmonella, Streptococcus, Staphylococcus* and *Campylobacteria*. Beneficial bacteria stimulate the gut epithelium to produce antimicrobial peptides and cytokines, including IL-12, IL-1β, IL-10, IFN-γ and TNF-α, enhance the synthesis of IgG, IgM and IgA and increase the number of macrophages and monocytes and natural killer cells, CD8, CD4 and CD3 T cells.

3.12 Effects of Stress on Meat Quality

Key concepts

- Pre-slaughter stress can increase the breakdown of muscle glycogen and production of lactic acid postmortem to decrease muscle pH and increase temperature.
- Stress results in negative effects on meat quality due to either PSE or DFD meat, depending on the glycogen content of the muscle.
- Porcine stress syndrome (PSS) is a genetic defect in the calcium release channel in muscle that can lead to PSE meat.
- Genetic tests have been developed for PSS but are not yet available for stress-susceptible poultry.
- Endocrine responses to stress include the SAM and HPA axis and thyroid hormones.
- Reducing stress prior to slaughter, rapid cooling of carcasses and treatment with electrolytes can reduce the incidence of PSE meat.

Pale, soft, exudative (PSE) and dark, firm and dry (DFD) meat

Pre-slaughter stress can alter post-mortem metabolism and result in meat quality issues, pale, soft, exudative (PSE) meat or dark, firm and dry (DFD) meat. Stress depletes muscle glycogen stores, which determines the glycolytic potential – the amount of lactate produced by post-mortem anaerobic glycolysis. For acute (short-term) stress immediately prior to slaughter this results in PSE meat, while chronic (long-term) stress can result in DFD meat.

PSE meat is a potential problem with poultry (chickens and turkeys) and pork. The PSE condition has been most extensively studied in pigs and can be caused by pre-slaughter stress from improper handling and mixing with unfamiliar animals; this induces a rapid post-mortem breakdown of muscle glycogen and subsequent increase in anaerobic glycolysis to form lactate. This increases the temperature of the muscle and dramatically decreases muscle pH, which denatures the sarcoplasmic reticulum proteins and decreases water-binding capacity of the tissue. An increased level of metabolites in the cells also increases the osmotic pressure, which causes water to move into the cells from the extracellular space. The muscle of pigs becomes pale, watery (exudative), sour-smelling and loose-textured. There is increased water (drip) loss and decreased water-binding capacity during storage, leading to decreased shelf life and poor consumer appeal and making it less suitable for further processing. The term PSE is a somewhat subjective description rather than an objective definition, so

different individuals' perceptions of what constitutes PSE can vary.

The water-binding capacity of meat is affected by pH. It is lowest at the isoelectric point of meat, between 5.0 and 5.1, since the protein has no net charge and the solubility of the proteins is at a minimum. Thus, as the pH of meat declines after slaughter, the water-binding capacity also decreases. In PSE pork, the exudate fills the spaces between the muscle fibre bundles and contributes to the soft texture of the pork. The paleness of meat is caused by the increased scattering of light within the meat due to protein denaturation. The pale colour of PSE pork takes at least 1.5 h to develop and the development of pale colour may continue for up to 4 days post-slaughter.

Stress can also cause DFD meat when levels of muscle glycogen are drastically depleted before slaughter, for example by chronic stress. This limits the amount of lactate that is formed post-mortem and causes a high ultimate pH in the meat at 24 h after slaughter. The high pH results in increased water-holding capacity, a firm texture and dry sticky surface of the meat. It also decreases meat pigment losses and denaturation, which increases light absorbance to produce an abnormal dark colour of the meat.

The colour of meat can be assessed objectively using a colorimeter (e.g. Hunter Laboratory MiniScan XE colorimeter and Minolta Chroma Meter CR-300 colorimeter). Spectral reflectance is determined over the 400–700 nm range and L, a and b, or L*, a* and b*, values are calculated from the Minolta or Hunter spectral curves, respectively. The L and L* values are measures of lightness; the a and a* values are measures of red colour; and the b and b* values are measures of yellow–blue colour. For DFD pork, longissimus muscle has a very dark colour (L values 35–45), while for PSE pork, the muscle has a very pale colour (L value 60).

The initial pH taken at 45 min after slaughter and the ultimate pH taken at 24 h after slaughter can be used to predict PSE and DFD meat (Fig. 3.40). A pork carcass with a pH of 6.0 at 45 min would have a rapid rate of glycolysis and a strong possibility of becoming PSE. At 24 h, normal pork has a pH of between 5.4 and 5.7; PSE pork can have a pH below 5.0 and DFD pork has a pH of greater than 6.0. For further details, see Matarneh *et al.* (2017).

PSE poultry meat is a problem, since it is unsuitable for further processing, due to excessive colour variation, defective water-holding capacity and poor binding ability, which reduce processed product yield and texture. PSE pork is also less suitable for the production of cured and processed products.

PSE pork was first described as a post-slaughter quality defect related to stunning technique, temperature of scalding water, duration of scalding and time until the carcass was chilled. A survey of the US pork industry in 2006 showed that 3.3% of loins exhibited all three conditions (pale, soft and exudative) of classical PSE, ranging from 0.1% to 10%. A survey of a pig slaughterhouse in Paraná,

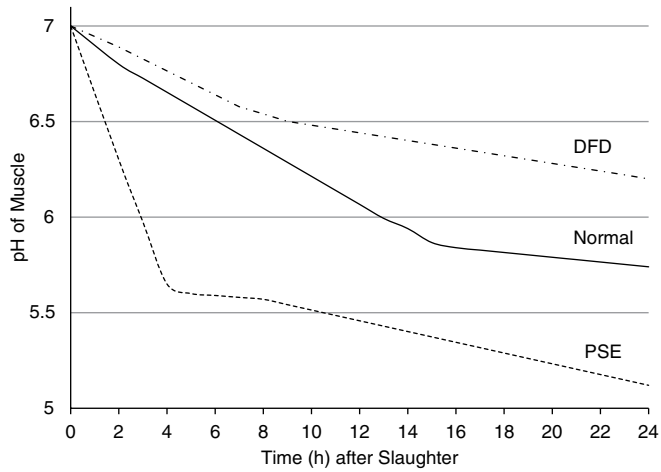


Fig. 3.40. Effects of muscle pH on DFD and PSE.

Brazil, in 2020 found the incidence of PSE (defined as pH less than 5.8 at 45 min after slaughter) was over 19%. The method of restraint and stunning and lairage time dramatically affects the incidence of PSE meat. Stunning with carbon dioxide produces the lowest incidence of PSE, followed by electrical stunning without restraint, with the highest incidence when electrical stunning with restraint is used. High-voltage (330–700 V) stunning increased PSE and blood splash compared with low-voltage (70 V) stunning. Carbon dioxide stunning is considered to be both humane and efficient and is increasingly used in slaughter plants. Handling of pigs in holding pens, truck-loading techniques, mixing of animals, crowding, temperature, travel time and access to feed and water are also important. Drip loss in PSE pork is about 1.70% of trimmed carcass weight, compared with 0.77% for normal pork, and drip loss increases with storage. The USDA has estimated that PSE meat costs the US pork industry US$30 million each year.

DFD meat is a problem with beef, pork and probably also with poultry. In beef, DFD meat is referred to as dark-cutting meat and is most severe in young bulls, due to their aggressive behaviour, which reduces muscle glycogen stores. The repletion of muscle glycogen during recovery from stress is typically a slow process, especially in ruminants fed a less energy-dense diet. In poultry there is some evidence that cyanosis is due to DFD meat. Cyanosis is the fourth leading cause of condemnation of chickens in Canada, and cyanotic meat is condemned because little is known about its safety and quality. It is thought to be caused by chronic hypoxia from overcrowding but may be related to general shipping stress. For more information about the effects of stress on meat quality, see the review by Gonzalez-Rivasa *et al*. (2020).

Porcine stress syndrome

Porcine stress syndrome (PSS) is a genetic defect that leads to malignant hypothermia (MH) in the live pig and the formation of PSE pork. The syndrome can be traced to early 20th century Germany and is particularly prevalent in the Piétrain breed. This problem may have begun by inadvertently selecting animals with the PSS mutation due to their exceptional muscle development in heterozygotes.

PSS pigs that are stressed develop hyperthermia (body temperature > 41°C) and take rapid, shallow breaths (dyspnoea). The skin becomes blotchy and the muscles tremble and become rigid or weak. The animal may die from acidosis, hyperkalaemia, vasoconstriction and cardiac arrest. Survival depends on cooling the animal and providing adequate oxygen. These effects are due to the rapid muscle metabolism, which increases oxygen consumption up to threefold and produces heat. The endocrine stress response is also stimulated (see below), which exacerbates the metabolic response.

In PSS pigs, the muscle pH can drop to 6.0 in less than an hour, due to excessive lactic acid accumulation, and the muscle temperature can rise to above 40°C, resulting in PSE meat. The DFD condition could be produced in PSS pigs that survived the initial stress response but were slaughtered with very low levels of muscle glycogen. This would result in low levels of lactic acid in the meat and an ultimate pH greater than 6.0.

PSS and PSE are not the same thing. PSS is a genetic defect of pigs while PSE is a non-specific meat condition that may occur in meat from an animal with accelerated post-mortem glycolysis. However, PSE meat is more prevalent in carcasses of pigs that suffer from PSS, with 60–70% of PSS pigs developing PSE meat, depending on post-mortem handling. Stress factors that can trigger PSS include breeding, high temperatures, crowding/mixing, transportation to the abattoir, vaccination, castration, moving within the abattoir and oestrus. Although PSS pigs are more susceptible to developing PSE meat, pigs that are free from the genetic defect can produce PSE meat as well. An examination of PSE loins for the PSS genotype showed that 3.6% were homozygous and 29% were heterozygous for the recessive stress gene, while the remainder were a normal genotype. This indicates that, while a high proportion of PSS pigs produce PSE meat, PSE meat can also be produced in normal pigs.

PSS is a genetic susceptibility to stress that is due to changes in calcium metabolism in the muscle (Fig. 3.41). Intracellular Ca^{2+} levels in relaxed muscle are 10^4 lower than in the extracellular fluid. During muscle contraction, Ca^{2+} that is stored in the sarcoplasmic reticulum in the muscle cell is released through a calcium release channel (CRC) (also known as the ryanodine receptor for its binding of the plant alkaloid, ryanodine) and intracellular

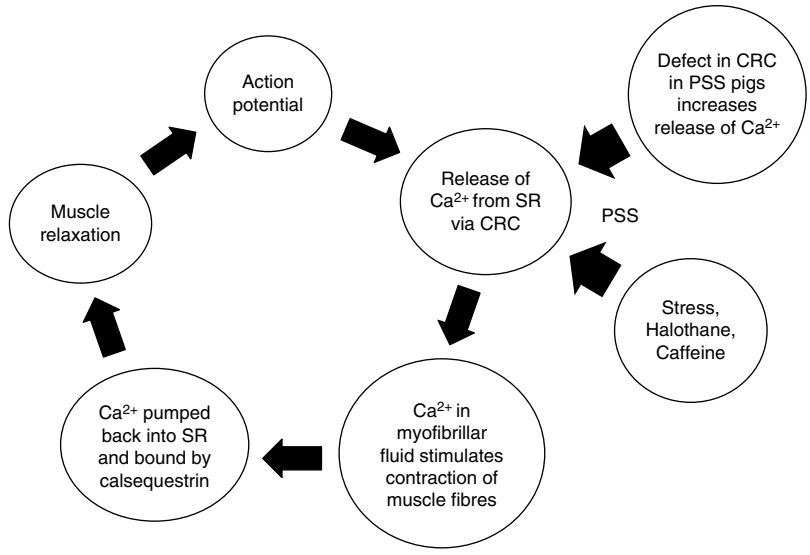


Fig. 3.41. Defect in calcium metabolism leading to porcine stress syndrome (PSS). CRC, calcium release channel; SR, sarcoplasmic reticulum.

Ca^{2+} is increased tenfold. Relaxation occurs as the Ca^{2+} is pumped back into the sarcoplasmic reticulum. The CRC is a large protein with a molecular mass of 565 kDa, which is activated by phosphorylation and is under negative feedback control from intracellular Ca^{2+}. It is also inactivated by calsequestrin (a protein that stores Ca^{2+} within the sarcoplasmic reticulum) and the action potential, which causes membrane depolarization and is measured by a voltage sensor that is physically connected to the CRC. Mg^{2+}, calmodulin and repolarization of the membrane, which is detected by the voltage sensor, inactivate the CRC. Non-physiological activators of the CRC include halothane, ryanodine and caffeine.

The PSS defect is due to the substitution of a single nucleotide (position 1843 of the *ryr-1* gene, HAL1843), which causes a single amino acid replacement (cysteine instead of arginine at amino acid 615, C615R) in the CRC. This SNP mutation causes the CRC to be more sensitive to biochemical activators, making it easier to open and more difficult to close. This, in turn, makes skeletal muscle easier to activate and more difficult to relax. A number of other mutations in the CRC have also been identified, but they are not as prevalent as the HAL1843 mutation.

A polymorphism in the dystrophin gene (*DMD*) that results in an arginine to tryptophan (R1958W) polymorphism in exon 41 of *DMD* has also been implicated in causing PSS (Nonneman *et al*., 2012). However, this has not been found to be widespread in commercial pig herds. A mutation has also been identified in the *PHKG1* gene that encodes a catalytic subunit of the phosphorylase kinase which functions in the activation of glycogen breakdown. This mutation results in decreased enzyme activity and a 43% increase in glycogen content of the muscle (Ma *et al*., 2014).

For interest

RN gene (Rendement Napole, Hampshire effect) is a dominant gene mutation that results in increased glycogen content of white muscle fibres. This results in low muscle pH, partial protein denaturation, decreased water-holding capacity and consequently low Napole yield (the process yield after curing in brine and subsequent cooking). It is an R200Q substitution in the *PRKAG3* gene (protein kinase, AMP-activated gamma 3 subunit). This is another example of effects of an AMP kinase.

Both the RN and HAL mutations have been mostly eliminated from commercial pig herds, so RN- and HAL-mediated reductions in pork quality have been reduced dramatically.

Testing for PSS

PSS pigs were first reliably identified by the halothane challenge test, which was developed in 1974, and the genetic component responsible was called the HAL gene. In this test, 2–3-month-old pigs are given 3–5% halothane in oxygen through a face mask and PSS-susceptible pigs develop extensor muscle rigidity when exposed to halothane gas. This is analogous to malignant hypothermia (MH) in humans. PSS is inherited as an autosomal recessive trait and the halothane challenge test is highly sensitive for homozygotes, but only 25% of heterozygotes are detected. The response in the test is increased if the pigs are stressed by fighting or rough handling. Skeletal muscle from PSS-susceptible pigs, as well as in malignant hyperthermia-sensitive people, is more sensitive to drug-induced contraction. This forms the basis for caffeine and halothane contraction tests using muscle biopsy specimens. The halothane challenge test does not work to screen out PSE-susceptible turkeys or chickens.

Following the development of the halothane challenge test, it was found that PSS was rarely found in Hampshire, Duroc, Large White and Yorkshire breeds, while it was prevalent in Piétrain, Landrace and Poland China breeds. The incidence of PSS found in Landrace pigs at different locations in the 1970s suggested that the defect spread from Germany and Belgium. Haplotype analysis of the *ryr-1* gene suggests that the PSS mutation arose from a single founder animal. It was found in virtually all domestic breeds used for intensive pork production worldwide and in more than 25% of the breeding stock in Europe and North America. In the early 1990s, the PSS mutation was found in 97% of Piétrain, 80% of Poland China, 37% of Landrace, 22% of Large White, Duroc and Hampshire and 17% of Yorkshire pigs. The spread of the mutation is due to the relatively small number of breeding pigs that are providing the genetics for the slaughter pigs, the introduction of new breeding stock in genetic improvement programmes and the fact that there is increased carcass lean in animals with the PSS mutation, which resulted in it being selected for in 'improved' genotypes. In North America, there was also no direct penalty to producers in the past for carcasses that have PSE.

Canadian researchers developed an early restriction fragment length polymorphism (RFLP)-based test that identifies the HAL1843 SNP mutation in pigs. DNA is first isolated from blood, muscle, semen or other tissues, with the preferred procedure being a dried blood sample on filter paper. A single hair bulb can also be used as a source of genomic DNA. A 659 bp portion of the *ryr-1* gene that includes the mutation is then amplified using gene-specific primers in the PCR, and the amplified DNA is subjected to RFLP analysis. This involves digesting the DNA with the restriction endonuclease BsiHKA I, which cuts the amplified DNA at a site common to both normal and mutant genes as well as at the mutation site itself. The number and position of DNA fragments that are found after agarose gel electrophoresis are used to identify the PSS genotype (O'Brien *et al*., 1993). SNP genotyping can now be done by more convenient and less expensive primer extension methods. This involves the addition of one base to a primer that anneals immediately *5′* to the SNP position and subsequent detection of the added base by fluorescent labelling or mass spectrometry (e.g. Carolino *et al*., 2007).

Two forms of the CRC in muscle (α-Ryr and β-Ryr) are present in avian species. Two forms of the *a-ryr* allele have been isolated, and turkeys homozygous for the *a-ryr* II alleles had superior meat quality. However, the point mutation identified in the *ryr-1* gene in pigs has not been found in poultry, and there are currently no reliable genetic markers for genetic selection against PSE meat in poultry. Other factors are probably responsible for the PSE condition in poultry, including the nature and metabolism of the breast muscles, the size of the muscle and its fibres, and environmental factors; in particular, heat stress that can cause accelerated post-mortem metabolism. Minimizing stress during handling and transport and optimizing chilling conditions post-mortem can reduce the incidence of PSE meat in poultry.

The development of technologies to identify the PSS mutation has reduced the severity and incidence of PSE in pigs but PSS has not been eliminated. The DNA test is an added cost and producers might not test their herds where there are no economic incentives. However, with the increased trend in 'branded products', meat quality has become more economically important. PSS pigs that survive to slaughter have higher lean yield, and a higher lean yield is also found in pigs that are heterozygous for the mutation. Thus, it may be advantageous to control the frequency of the mutation and to raise heterozygous animals rather than to completely eliminate it.

The PSS mutation results in improved carcass characteristics and feed conversion in heterozygous animals. Landrace pigs that are heterozygous for the mutation have 5% less back fat and 2.8% increased lean yield, while heterozygous Yorkshire pigs have 5.7% improved feed conversion, 6.3% decreased back fat and 4.1% increased lean yield compared with non-mutant pigs. The increased lean and decreased fat may be a direct consequence of the increased sensitivity of the muscle to contraction and increased metabolic activity of the muscle in heterozygous pigs. This may act directly as a stimulus for muscle hypertrophy and mobilization of fat in PSS pigs.

The *ryr-1* gene is also expressed in brain, while two other CRC genes, *ryr-2* and *ryr-3*, are expressed in smooth muscle and brain. The PSS mutation does not, therefore, affect cardiac or smooth muscle. The presence of the PSS mutation in the brain may mean that the central nervous system is more sensitive to stress in mutant animals. Regional differences in levels of neurotransmitters in the brain have been reported in affected pigs but these may be secondary effects from the altered muscle metabolism.

PSS is analogous to MH found in humans, dogs and horses. Although MH is due mainly to a single mutation in pigs, there are many different mutations that can cause MH in humans. MH is also 5000- to 25,000-fold less common in humans than in pigs. This is because modern domestic pigs are highly inbred and the PSS mutation has probably been selected for due to the improvements in carcass composition and feed conversion associated with it.

Endocrine factors that affect PSS pigs and PSE pork

During stress, secondary responses of the endocrine system occur with epinephrine and cortisol (see also Section 6.3 for a discussion of stress). The adrenal catecholamine epinephrine is synthesized from norepinephrine in the adrenal medulla. The catecholamines act as short-term response hormones to cause vasoconstriction and other changes for the 'fight or flight' response. There are both α-adrenergic receptors, which lead to vasoconstriction, and β-adrenergic receptors, which stimulate vasodilation (see Section 3.7). Arterial and venous systems contain mostly α-receptors, while skeletal and cardiac muscles contain both α- and β-receptors. Norepinephrine stimulates α-receptors and causes vasoconstriction, while epinephrine stimulates both α- and β-receptors. The overall vascular response of an organ to catecholamines thus depends on the relative amounts of epinephrine and norepinephrine and the ratio of α- to β-adrenergic receptors present. In skeletal muscle, high levels of catecholamines result in vasoconstriction.

During the stress response (Fig. 3.42), epinephrine levels in PSS pigs rise quickly and epinephrine acts to

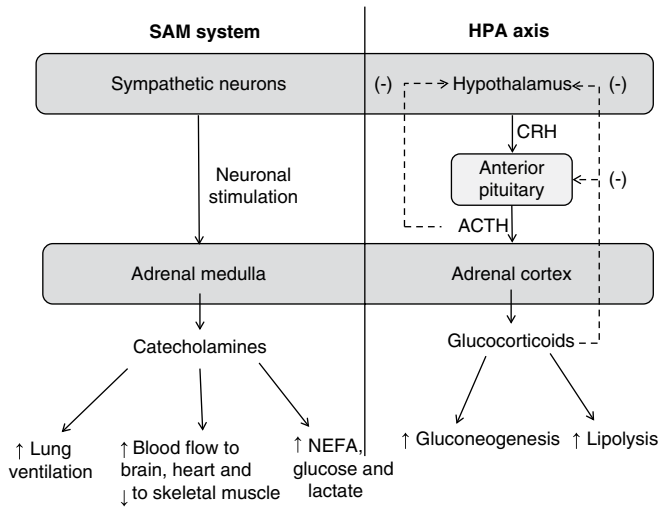


Fig. 3.42. Hormonal and metabolic responses to stress.

increase the activity of glucagon. Glucagon and epinephrine work via a cAMP-dependent pathway to stimulate glycogenolysis, which is the breakdown of glycogen stores. Glycogen is converted to glucose-6-phosphate during glycogenolysis; and during glycolysis, glucose-6-phosphate is converted to pyruvate. After the pig is slaughtered, pyruvate is converted to lactate under anaerobic conditions. Lactate builds up in muscle to cause a dramatic decrease in muscle pH, leading to PSE pork.

Epinephrine causes vasoconstriction in muscle and a hypoxic condition results in the muscle in PSS pigs, due to the lack of oxygenated blood. This causes lactate to accumulate from anaerobic glycolysis. In a live pig under anaerobic conditions, the excess lactate would be shunted in the blood to the liver and be converted back to glucose; this is known as the Cori cycle. Under aerobic conditions, pyruvate from glycolysis is converted to acetyl-CoA, which enters the TCA cycle.

The glucocorticoid cortisol is produced in the adrenal cortex and is a long-term stress hormone. Its major target tissue is the liver; other important target tissues are the lymphoid cells, thymus gland and kidney. Cortisol affects carbohydrate metabolism by promoting gluconeogenesis from body proteins, enhancing fatty acid mobilization and oxidation, and increasing plasma cholesterol and triacylglycerol levels and glycogen deposition in the liver. This affects water and electrolyte distribution in tissues by opposing water shift into the cells.

Gonadotrophin secretion is also affected, as cortisol inhibits GnRH stimulation of LH release. In the gastrointestinal tract, prolonged high levels of cortisol encourage the development of ulcers. The inhibition of prostaglandin synthesis is possibly involved in this process. Cortisol also has effects on immunity and inflammation (see Section 6.3). Cortisol is involved in placental endocrine function. Maternal cortisol levels are higher and the placenta contains cortisol receptors. Cortisol promotes the secretion of chorionic gonadotrophin, which is a hormone required for the maintenance of pregnancy. However, increased production of cortisol by the fetus acts as a trigger for parturition (see Section 5.1).

The effects of cortisol are more pronounced in PSS pigs. When the pig is stressed, cortisol levels rise. Because of the problems with calcium sequestration in PSS pigs, the animal's muscles continue to contract. Cortisol levels remain high for extended periods and this increases gluconeogenesis from body proteins. When a pig is stressed during transportation or at the abattoir, cortisol levels will be high. Cortisol has permissive effects on catecholamines; therefore epinephrine will more readily convert glycogen to glucose. Upon death, glucose will be utilized by anaerobic glycolysis to produce lactate in the muscles, leading to PSE meat.

Thyroid hormones may also play a role in the development of PSE meat. Thyroid hormones normally stimulate heat production by increasing aerobic metabolism. Removing the thyroid glands from pigs decreases the rate of postmortem glycolysis, while supplementing pigs with thyroid hormones increases the rate of pH decline in the muscles. For more information, see Matarneh *et al*. (2017).

Manipulations to reduce the incidence of PSE

Studies have shown that the mutation at position 1843 of the *ryr-1* gene accounts for only 25–35% of the PSE meat observed in commercial abattoirs, so methods other than genetic selection are required to reduce the general incidence of PSE. Reducing stress prior to slaughter will reduce the incidence of PSE meat. Adequate pen space should be provided in the holding pens at the slaughter plant (0.6 m^2 per 115 kg pig). All pigs must have room to lie down and during hot weather the animals can be wetted with sprinklers. Prior to stunning, 2–4 h of rest should be allotted. Animals should be handled and driven quietly, with minimal electric prod usage. Overcrowding and exposure to unfamiliar animals should be avoided. The animals should have room to turn so they can enter the race more easily. Pigs must always have access to water. Supplementing animals with electrolytes and feeding a high-quality protein such as casein may also reduce the stress response.

Rapid cooling of the carcass would reduce the formation of PSE conditions, since it would reduce the denaturation of sarcoplasmic proteins caused by the excessive heat from the rapid metabolism. Injection of sodium bicarbonate has been shown to reduce the rate or extent of post-mortem pH decline. Injection of 10% by weight of 0.3 M sodium bicarbonate at 15 min after death improved colour, reduced drip loss and increased the ultimate pH of longissimus and biceps femoris muscles. Including 0.7% NaCl along with the sodium bicarbonate improved juiciness and flavour. Injection at 24 h after death improved drip loss but not the colour defect. Selective breeding for high ultimate

pH would improve water-holding capacity, colour and tenderness of meat. For more information on approaches to reduce the incidence of PSE in pork and poultry, see Barbut *et al.* (2008) and Dong *et al.* (2020).

Summary and Conclusions

In this chapter, a number of methodologies have been described to manipulate the growth rate, carcass composition and meat quality of meat animals. Some of these methods, such as the use of anabolic steroids and antimicrobials, have been used for many years to improve growth rate, performance and lean yield. The use of uncastrated male pigs has also been used for a long time in a few countries but has yet to be a universally accepted procedure for pig production. Other technologies, such as the use of somatotrophin or β-agonists and reducing the incidence of PSS, have been used for the past number of years. Other endocrine systems, such as leptin and adipokines, control of appetite and use of various dietary PUFAs, are being considered for use.

A thorough understanding of the underlying biology is necessary to develop effective methods to manipulate these endocrine systems. In a number of cases, this has allowed the development of specific receptor agonists or antagonists that affect only the appropriate components of the endocrine system to produce the desired results without adverse side effects. Other applications are to use genetic markers in endocrine-related genes in breeding programmes to generate lines of animals with desirable traits.

Questions for Study and Discussion

Section 3.1 Overview

1. Outline the hormones involved in regulating (i) growth and (ii) body composition.
2. Describe the metabolic problems that are the result of selection for increased muscle mass in livestock.
3. Describe the process of myogenesis to increase muscle mass.
4. Describe the process of adipogenesis and the structure of adipose tissue. What are the key metabolic roles of adipose tissue?
5. Describe the structure of bone and the process of bone formation and resorption.

Section 3.2 Anabolic steroids

1. Describe the use of anabolic steroids in meat animal production and in horse racing.
2. Describe methods to distinguish between anabolic and androgenic effects of various steroids. Give examples of structural changes that have been used to optimize these effects.
3. Describe the direct and indirect mechanisms of action of anabolic steroids.
4. Describe a mechanism to explain the tissue-specific effects of anabolic steroids.
5. Discuss potential safety issues with the use of anabolic steroids.

Section 3.3 Use of intact male pigs

1. Discuss the advantages and disadvantages of raising intact male pigs for pork production.
2. Describe the role of androstenone and skatole in causing boar taint.
3. Describe the metabolism of boar taint compounds androstenone and skatole.
4. Outline analytical and potential on-line methods to measure boar taint.
5. Discuss potential methods to control boar taint.
6. What are the limiting factors preventing the universal use of entire male pigs for pork production?

Section 3.4 Somatotrophin

1. Outline the effects of ST on growth, carcass composition and meat quality.
2. Discuss the control of pulsatile ST release. Describe how ghrelin and GHRH control ST release.
3. Comment on the role of ST in the ageing process
4. Describe the direct metabolic effects of ST.
5. Describe the indirect effects of ST via IGF-1.
6. Outline potential methods to manipulate the effects of ST.

Section 3.5 Adipokines

1. Outline the contrasting roles of leptin as an 'adipostat' and adiponectin as a 'starvation signal' in regulating feed intake and energy expenditure.
2. Describe the mechanism of action of leptin. Give an example of how leptin is being exploited to optimize carcass composition.
3. Describe the effect of leptin on heat generation. What other endocrine systems are also important in this function?

4. Describe the role of leptin in regulation of reproduction. Outline the role of AMPK in the metabolic effects of leptin and adiponectin
5. Explain why obesity is considered to be a condition of chronic inflammation. How does this affect metabolism?
6. Describe the proposed role of resistin in interfering with insulin signalling pathways.
7. Describe the proposed role of irisin to increase energy expenditure and thermogenesis.

Section 3.6 Thyroid hormones

1. Describe the synthesis and metabolism of thyroid hormones.
2. How is the activity of thyroid hormones regulated in different tissues?
3. Describe the mechanisms of action of thyroid hormones.
4. Describe the role and mechanism of action of the thyroid hormone metabolite, 3-iodothyronamine (T1AM).
5. Discuss the metabolic roles of thyroid hormones.
6. Describe the role of thyroid hormones in growth.
7. Thyroid hormones are involved in diverse functions such as metamorphosis in amphibians, brain development and feather growth. Explain.

Section 3.7 β-Adrenergic agonists

1. Describe the role of the sympathetic nervous system and catecholamines in regulation of metabolism.
2. Describe the types of β-AA receptors and their interaction with various β-AA compounds. What are the important structural components of β-AA that confer increased activity?
3. Outline the metabolic effects of β-AA and their mechanism of action that result in changes in carcass composition.
4. Why are the effects of some endocrine factors that affect growth and carcass composition additive?
5. Comment on the safety concerns for β-AA use.
6. What are the advantages of β-AA versus ST use?

Section 3.8 Dietary chromium and insulin

1. Describe the role of chromium and the potential mechanism of chromodulin in modulating insulin activity.
2. Outline the potential physiological roles of dietary chromium. What are the potential benefits in different species of livestock?
3. Why would chromium be potentially more effective in livestock than in humans?
4. Discuss the effective dose and potential safety concerns of dietary chromium.

Section 3.9 Dietary PUFAs

1. Discuss the effects of PUFAs and their metabolites on lipid and carbohydrate metabolism.
2. Describe the mechanisms of regulation of gene expression by PUFAs.
3. Comment on the interactions between the metabolism of *n*-3 and *n*-6 PUFAs.
4. Describe the metabolic pathways of LA, ALA and GLA leading to eicosanoid production.
5. How are industrial *trans* fatty acids produced and how do they differ from ruminally derived PUFA with a *trans* double bond? What are the effects of industrially derived *trans* fatty acids on metabolism and health?
6. Outline the effects of CLA isomers on metabolism and carcass composition.
7. Describe the potential applications of dietary PUFAs.
8. Describe some of the challenges in supplementing animal products with desirable PUFAs.

Section 3.10 Regulation of food intake

1. Outline how the gut–brain axis works to regulate feed intake.
2. Describe the role of anorexigenic and orexigenic neurons in regulating appetite.
3. What are 'satiation signals' and 'adiposity signals' and how do they contribute to regulate feed intake and body composition?
4. Outline the endocrine responses to fasting and feeding.
5. Propose a novel method to: (i) increase feed intake; and (ii) decrease feed intake. Give an example of when each of these methods could be used in animal production.

Section 3.11 The Gut Microbiome and Antimicrobials

1. Describe the interaction between the gut microbiome and the host animal. How does the microbiome affect the health and productivity of the host animal?
2. Describe methods to evaluate the composition of the microbiome. How can the composition of the microbiome be altered to benefit the host animal?

3. Describe the prophylactic use of antimicrobial compounds to improve animal production. What are the problems associated with this practice? What alternatives to antimicrobial use are available?
4. Describe methods to control methane production in ruminants. Why is this important?
5. How can the microbiome be manipulated to control post-weaning diarrhoea in piglets?
6. Describe the use of prebiotics and probiotics in poultry.

Section 3.12 Effects of stress on meat quality

1. Describe the mechanisms of how stressing an animal can lead to poor meat quality.
2. Discuss the post-mortem factors that lead to the development of PSE and DFD meat.
3. Describe genetic defects leading to PSS and testing procedures for PSS-susceptible pigs. What is the advantage of not eliminating this mutation?
4. Why is PSS more frequent than malignant hypothermia in humans? Give an example of another spontaneous mutation that affects carcass composition in livestock.
5. Discuss the endocrine responses in the HPA and SAM axes that lead to PSE.

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4 Endocrine Effects on Animal Products

This chapter deals with hormonal systems that can affect the production of animal products other than meat, namely, milk, eggs and wool. The production of milk is covered first, including mammary gland development, the initiation and maintenance of lactation, factors affecting milk composition, bioactive compounds in milk and metabolic diseases of lactation. The section on egg production covers endocrine factors that regulate sexual differentiation and development, the regulation of follicular development and egg production, the manipulation of moulting and the formation of the eggshell in poultry. The section on wool production starts with a description of skin as an endocrine organ and then the various dietary and endocrine manipulations that can be used for defleecing sheep are described. The endocrine factors (cytokines) that are involved in the function of wool and hair follicles, in particular the epidermal growth factors, are discussed.

4.1 Mammary Gland Development and Milk Production

Key concepts

- The lactation cycle is regulated by reproductive hormones, metabolic hormones and hormones produced by the mammary gland.
- The number of milk-synthesizing cells is the major factor that determines milk yield.
- Mammary gland development (mammogenesis) is related to reproductive development and is dependent on oestrogen and progesterone, along with ST and prolactin (PRL).
- The initiation of lactation (lactogenesis) is stimulated by PRL, oestrogen and glucocorticoids.
- The maintenance of lactation (galactogenesis) is controlled by PRL, ST and T_3 and milk removal.
- Exogenous administration of bST increases milk yield in dairy cattle.
- *In vitro* cell culture and whole-animal model systems have been developed to study mammary gland function and development.
- The composition of milk fat can be readily altered by diet, with lesser effects on milk protein.
- Mammary secretions (milk and colostrum) provide nutrients (lipids, carbohydrates and proteins) and bioactive compounds to promote growth and development.
- Metabolic diseases of lactation can occur due to high metabolic demands at the initiation of lactation when the cow is in negative energy balance. Ketosis results from a high demand for glucose, and milk fever is due to a severe drop in blood calcium.

Introduction

The endocrine factors that affect milk production are important in the growth and development of the mammary gland (mammogenesis), the initiation of lactation (lactogenesis) and the maintenance of lactation (galactopoiesis). Metabolic diseases related to lactation, such as ketosis and milk fever, are also affected by endocrine factors. Most of the material in this section is related to dairy cows, with differences found in other species noted.

The mammary gland or udder is a skin gland located outside the abdominal cavity. In cows, the right and left sides of the udder are entirely separate, with no common blood supply. There is also no internal crossover of the milk duct system among the individual udder quarters, so substances injected into the teat and duct system of one quarter will only be found in that quarter. Sheep and goats have two separate udder halves but do not have the four quarters of the udder that cows have. Pigs have 6–7 rows of paired teats for a total of 12–14 teats, with each teat connected to two separate udder halves.

Milk is synthesized and stored in parenchymal tissues of the mammary gland. Milk-secreting cells

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are organized in small sack-like structures called alveoli; groups of alveoli are organized into lobules; and groups of lobules are organized into lobes (Fig. 4.1). The epithelial lining of the alveolus is surrounded by myoepithelial cells, which contract in response to the hormone oxytocin to squeeze the milk out into small ducts. The small ducts connect with larger ducts, which deliver the milk to the cistern within the gland for storage. There is no direct innervation of the secretory system to effect milk delivery. In response to stimulation of the teat, oxytocin is released from the posterior pituitary gland (see Section 1.4), which induces a milk ejection reflex to move milk from the alveoli into the cistern of the udder.

Model systems for studying mammary gland function

Whole-animal studies

Mammary gland function can be assessed by biopsy of mammary tissue from the live animal or by imaging the mammary tissue using magnetic resonance imaging (MRI). In a unilateral frequent milking (UFM) model, different sides of the udder can be milked at different frequencies to study the effects of milking frequency on metabolism and milk production. Performing studies within the same udder decreases variability due to genetic factors, the environment, or nutrition.

An *in vivo* model for studying endocrine effects on the mammary gland is the method of close arterial infusion into the mammary gland (see Fig. 2.7) (Maas *et al*., 1995). This can be accomplished without general anaesthesia or deep surgical dissection, which minimizes the stress to the cow. The arterial supply to the two contralateral sides of the mammary gland can be catheterized separately, so that one side of the gland can be used for the treatment and the other side for the control. The catheter is placed into the saphenous artery and inserted up to the external iliac artery, which supplies the ipsilateral mammary glands via the external pudendal artery and the hind limb via the femoral artery. The position of the catheter is determined by real-time ultrasound imaging. Evans blue dye injected through the catheter appears exclusively in the ipsilateral vein.

Test compounds can also be infused into one half of the udder via the streak canal in the teat. The contralateral half of the udder is the control and receives the vehicle alone. This method tests the activity of compounds that are infused into the internal milk secretory system of the udder rather than delivered to the mammary gland via the blood.

In vitro *cell culture systems*

Bovine mammary epithelium cells can be isolated and cultured in a collagen matrix to form three-dimensional structures resembling alveoli

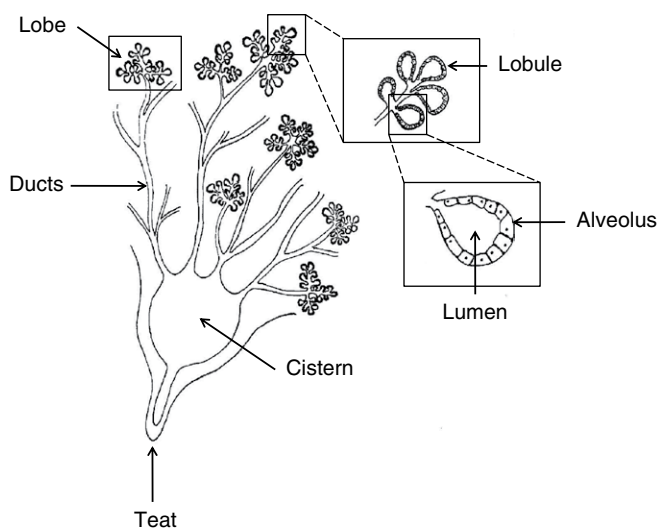

Fig. 4.1. Diagram of mammary gland structure.

(see Section 2.1). Mammary tissue can be obtained from pregnant cows, minced and dispersed in a mixture of collagenase and hyaluronidase. The epithelial cells are then purified by density-gradient centrifugation. Alternatively, a cloned mammary cell line can be used, such as the MAC-T3 (mammary alveolar cell transfected with large T antigen). The matrix in which the cells are grown affects mammary cellular functions. The basement membrane is a component of the extracellular matrix and contains proteins such as collagen, laminin and proteoglycans. Epithelial cells are in contact with the basement membranes, which allows the cells to develop polarity and secrete milk components at the apical surface of the cell.

Mammary gland development (mammogenesis)

The number of milk-synthesizing cells is one of the major factors that determine the milk yield capacity of the mammary gland and the shape of the lactation curve (see Fig 4.6). Factors that affect mammary development during rearing can affect the number of milk-producing cells and thus affect the future milk yield capacity of the mature cow. The milk yield of sows on a per kilogram body weight (BW) basis is comparable to that of dairy cows. However, milk production in sows can potentially limit piglet growth, since hyperprolific sows may have been selected for larger litter size without an increase in milk production.

Mammary gland development is related to reproductive development, which occurs in distinct phases during fetal life, puberty, pregnancy and lactation (Fig. 4.2). At birth, the non-epithelial tissues and basic structures of the mammary gland, including the connective tissue and blood and lymph vessels, have already formed. However, the secretory and glandular tissue is still rudimentary. From 2–3 months of age until shortly after puberty is completed in cattle, the mammary gland undergoes an allometric growth phase, in which it grows faster than other parts of the body. During this period, the fat pad and mammary ducts grow rapidly but no alveoli are formed. At puberty, the mammary glands of heifers weigh about 2–3 kg, with 0.5–1 kg of parenchymal tissue, which consists of 10–20% epithelial cells, 40–50% connective tissue and 30–40% fat cells.

At birth, each mammary gland of the piglet is composed of the teat, including the thick connective

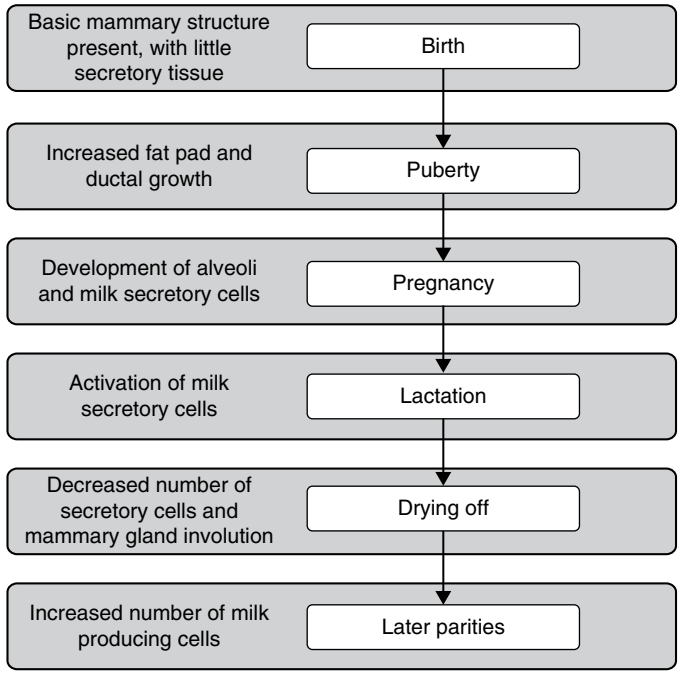

Fig. 4.2. Mammary gland changes during development.

tissue base of the teat, a fat pad and connective tissue, two lactiferous ducts, and a few ducts branching into the fat pad. Growth of mammary gland parenchyma and fat pad is slow until about 90 days of age, followed by a four- to sixfold increase in mammary DNA until puberty, with parenchymal tissue mass increased by 51% in gilts that have reached puberty.

Increased growth rate due to feeding high-energy diets near puberty can lead to permanently reduced milk yield potential. This is due to excess fat deposition in the mammary gland and decreased growth of parenchyma. This may not occur when a high-energy and high-protein diet is fed, so that dietary protein does not limit mammary gland development. Higher BW gain after puberty and during pregnancy has no effect on mammary growth and potential milk yield.

Very little mammary growth occurs after puberty until pregnancy. In early pregnancy, the mammary ducts grow and there is extensive lobulo-alveolar development from mid-pregnancy. The growth rate is exponential, with most of the growth occurring in the last trimester of pregnancy. The growth and development during this period determine the number of milk-secreting cells and thus the potential for milk production. Mammary epithelial cells grow into the fat pad and the developing structures will only be formed when the fat pad is present. This may involve specific fatty acids from the fat pad. Cell numbers continue to increase even after parturition. Increased milking intensity increases mammary growth during lactation. The lactating mammary gland in cattle weighs 15–25 kg, with the parenchyma consisting of 40–50% epithelial cells as ducts and alveoli, 15–20% lumen, 40% connective tissue and almost no fat cells. Mammary glands of pigs also undergo major changes during the last trimester, when the adipose and stromal tissues are extensively replaced by alveolar tissue that produces milk. Glands in the thoracic region are largest and produce the most milk, followed by the glands in the mid region, with the inguinal glands the smallest. Feed restriction decreases mammary parenchymal growth during this period.

About 3–4 weeks prepartum, the mammary gland begins the transition from the non-lactating to the lactating state. Transport of IgG into the colostrum begins 2 weeks prior to parturition, and the concentrations of the major milk components increase dramatically 3–5 days prepartum. During lactogenesis, the alveolar secretory cells are activated. The amount of rough endoplasmic reticulum, Golgi and mitochondria increases in alveolar cells. The activities of enzymes associated with lactation, such as acetyl-CoA carboxylase and fatty acid synthase, are increased, along with the transport systems of the substrates for milk synthesis, including amino acids and glucose. Casein micelles and fat droplets accumulate in the cytoplasm and are released into the lumen of the alveolus. There is a two- to sixfold increase in blood flow to the mammary gland, starting 2–3 days postpartum. However, the decrease in production that normally occurs later in lactation is not due to decreased blood flow; it is due to a decrease in the number of milk-secreting cells.

In gilts, the third stage of rapid mammary development is during lactation. The average weight of suckled mammary glands increases linearly from 380 g on day 5 of lactation up to 590 g on day 21. Feeding in gestation affects body condition, and gilts that are either too fat (36 mm backfat) or too lean (12–15 mm backfat) at the end of gestation have less developed mammary tissue.

Mammary duct development increases by around 8% in each oestrous cycle and then regresses during metoestrus and dioestrus. After the first pregnancy, cell numbers can be higher for subsequent lactations. Milk yield increases until the cow is 8 years old, with mature cows producing 25% more milk than 2-year-old heifers. However, the persistency of lactation is higher in primiparous than in multiparous cows. Mammary development is also affected by parity in sows, as mammary gland wet weight increases by 70%, 20% and 30% between day 113 of gestation and day 26 of lactation, for sows of parity 1, 2 and 3, respectively.

Involution and the dry period

As lactation progresses in dairy cattle, the number of mammary cells decreases due to apoptosis, related to decreasing levels of ST, prolactin and IGF-1, resulting in decreased milk yield (see Fig. 4.6). Pregnancy also decreases milk yield, possibly due to the effects of oestrogen produced by the placenta on mammary function. The decrease in milk yield eventually results in an end to lactation and mammary gland involution. Lactating cows are usually dried off 8–9 weeks before planned parturition. In contrast with other milk proteins, the synthesis of lactoferrin is increased during involution; lactoferrin may act as a non-specific disease-resistance

factor. The level of the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase also increases in the mammary gland during involution. There is a decrease in the size of the alveoli and lumen during involution, but general alveolar structure is maintained in the bovine mammary gland. In rodents, there is extensive tissue degeneration and disintegration of the alveolar structure through apoptosis during mammary gland involution. When piglets are weaned or when a gland is not suckled, those mammary glands in sows undergo a process of involution with a very rapid regression occurring during the first 7 days after weaning and a loss of two-thirds of parenchymal tissue.

A dry period is required between the end of lactation and calving so that milk yield in the next lactation will not be reduced. To induce the dry period, the amount of grain and water supplied to the cow is first limited to reduce milk production, and then milking is stopped 45–50 days before the expected date of parturition. A shorter dry period significantly reduces subsequent milk production. The early dry period is the time of the highest incidence of new intramammary infections. This may be due to the presence of a large volume of milk in the gland, which is conducive to bacterial growth, leakage from the teats or the lack of teat-end disinfection. The concentration of total leukocytes increases in mammary secretions during this period.

Hormones and mammary gland development

Three categories of hormones are involved in the lactation cycle. The reproductive hormones (oestrogen, progesterone, placental lactogen, prolactin and oxytocin) act directly on the mammary gland. The metabolic hormones (ST, glucocorticoids, thyroid hormone, insulin and hormones from the GI tract) indirectly regulate milk production by altering nutrient flux to the mammary gland and may alter the responsiveness of the mammary gland to reproductive hormones. The mammary gland also produces hormones, including ST, prolactin, parathyroid hormone-related peptide (PTHrP) and leptin. The autocrine and paracrine factors important in the three stages of mammary growth and development are listed in Table 4.1.

The hormones involved in mammogenesis are illustrated in Fig. 4.3. Oestrogen, acting through the ERα, stimulates mammary duct growth, and oestrogen and progesterone act synergistically to stimulate lobulo-alveolar development. Progesterone is elevated throughout gestation and oestrogen is particularly elevated during the latter half of pregnancy. Thus, mainly ductal and lobule growth occur during the first half of pregnancy, with lobulo-alveolar growth occurring during the second half of pregnancy. Relaxin is secreted by the corpora lutea in pigs and works synergically with oestrogen and progesterone to stimulate mammary growth and development in sows.

Table 4.1. Hormone effects on mammary development and milk production.

Stage of development	Stimulated by	Inhibited by
Mammogenesis	Oestrogen/ progesterone, prolactin, placental lactogen, ST/ IGF-1, EGF, FGF, TGFα, MCSF, HGF, Cripto-1, relaxin	MDGI, TGFβ
Lactogenesis	Prolactin, oestrogen, glucocorticoids, insulin, leptin, GI tract hormones	Progesterone
Galactopoiesis	Thyroid hormones, prolactin, milk removal, ST	Glucocorticoids, oestrogen

Prolactin and growth hormone (ST) are needed for the steroid hormones to be effective. Prolactin binds to its receptor on the mammary epithelial cell, which induces conformational changes in the receptor and activates JAK2. JAK2 phosphorylates and activates members of the signal transducer and activator family of transcription factors (STAT5) (see Section 1.3), which then activate certain genes involved in differentiation of terminal end buds of the mammary ducts. Levels of prolactin, ST and insulin decrease during gestation.

Placental lactogen is a peptide hormone produced by the placenta and is structurally related to prolactin and/or ST, depending on the species. It binds to the prolactin receptor in rodents to stimulate mammogenesis during pregnancy. Levels of placental lactogen in the cow are very low in maternal blood, so it has little effect in cattle.

Mammary-derived growth inhibitor (MDGI) is a 14.5 kDa protein that is produced by mammary epithelial cells. It acts in an autocrine manner to inhibit cell growth and especially to induce differentiation in mammary epithelium. MDGI levels increase in the mammary gland 2 weeks prior to

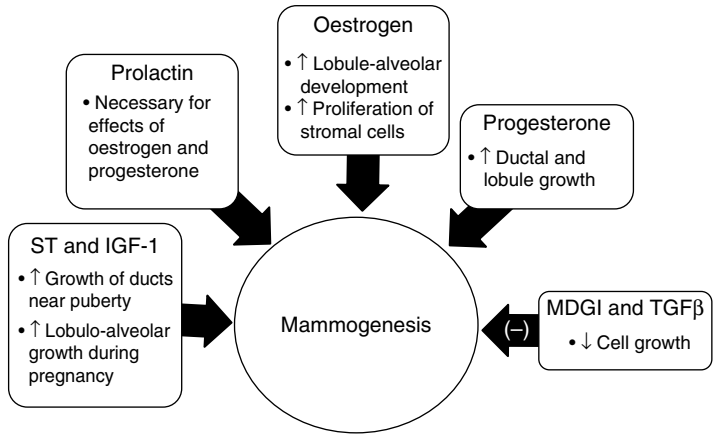

Fig. 4.3. Hormones involved in mammogenesis in cattle.

parturition and are high in lactating dairy cows. MDGI is the same as the fatty acid-binding protein isolated from heart (H-FABP). FABP is important in intracellular transport and metabolism of fatty acids, cell differentiation and signal transduction.

Epidermal growth factor (EGF) stimulates mammogenesis, but not in cattle. Fibroblast growth factors (FGFs) may be involved in the growth of mammary stromal cells such as fibroblasts. Transforming growth factor-α (TGFα), hepatocyte growth factor (HGF) and macrophage colony-stimulating factor (MCSF) have also been implicated in stimulating mammogenesis. TGFβ inhibits mammogenesis in mice and may also be effective in cattle. See Section 4.3 for more information on the EGF family of growth factors.

ST stimulates the growth of ducts during mammary development near puberty and lobulo-alveolar growth during pregnancy. Injection of ST between 8 and 16 months of age increases growth of the parenchyma and total mammary cell numbers in cattle. This occurs via the action of IGF-1 produced either by the liver or locally in the mammary stroma. The negative effects of high feeding level on mammary growth near puberty may be due to increased local production of IGFBP-3, which binds IGF-1 to inhibit it.

Oestrogen stimulates proliferation of stromal cells in the mammary gland and stromal cells produce IGF-1. Insulin has little effect on mammogenesis *in vivo*, but administration of very high levels of insulin can mimic the effects of IGF-1. Cortisol targets the endoplasmic reticulum and Golgi apparatus in the differentiation of the lobulo-alveolar system in cattle. This is necessary so that prolactin can later induce the synthesis of milk proteins. For a review of hormonal effects on mammary growth and lactation, see Farmer (2013) and Hurley (2019).

Hormones and initiation of lactogenesis

There is considerable species variability in the effects of hormones on lactogenesis (the start of milk production) and galactopoiesis (the maintenance of milk production). These hormones are outlined in Figs 4.4 and 4.5.

Prolactin, oestrogen and glucocorticoids initiate lactation, provided that there is a well-developed lobulo-alveolar system. Oestrogen stimulates the release of prolactin from the anterior pituitary and increases the number of prolactin receptors in mammary cells. There is a surge of prolactin several hours before parturition. If this surge is blocked with bromocriptine, milk yield is reduced. Prolactin increases the translation of milk protein mRNAs, swelling of Golgi membranes and milk protein secretion, along with synthesis of lactose and milk fat. Prolactin also increases the uptake of calcium and long-chain fatty acids from the intestine. Leptin (see Section 3.5) and hormones of the GI tract (see Section 3.10) regulate feed intake and nutrient absorption.

Injections of glucocorticoids into non-lactating cows with well-developed lobulo-alveolar systems induce the onset of lactation. Milk production is increased if prolactin is also present, and there is a synergy between glucocorticoids and prolactin in initiating lactation. Glucocorticoids bind to recep-

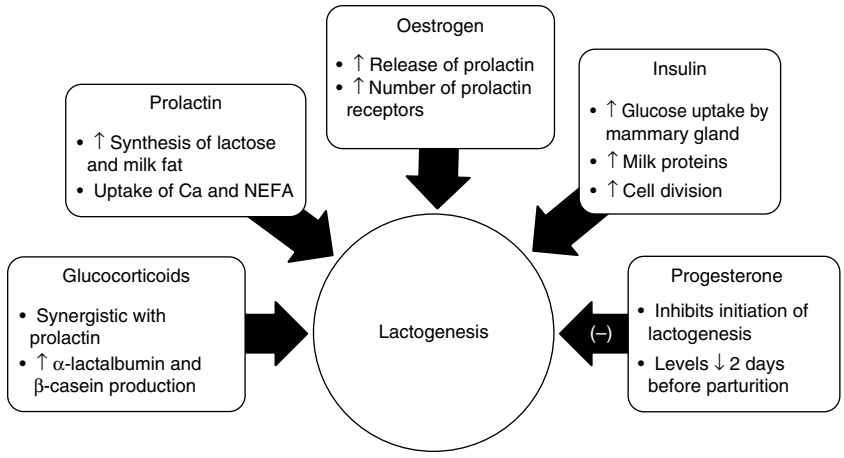

Fig. 4.4. Hormones involved in lactogenesis.

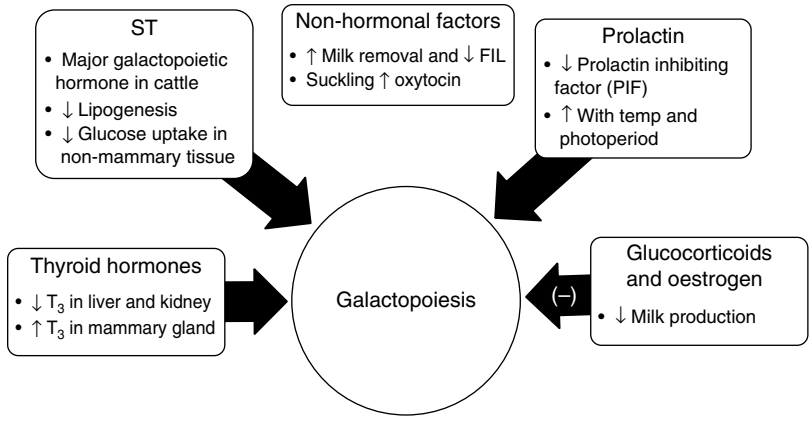

Fig. 4.5. Factors involved in galactopoiesis.

tors in mammary tissue to increase the development of the rough endoplasmic reticulum and other ultrastructural changes to increase the secretion of α-lactalbumin and β-casein. Binding to the corticosteroid-binding globulin (CBG) reduces the activity of glucocorticoids in serum. During the periparturient period, levels of the CBG decrease and free glucocorticoid levels increase, which might explain the lactogenic effects of glucocorticoids. Glucocorticoids also suppress the immune system, which may contribute to the increased incidence of mastitis during early lactation.

Insulin is important in stimulating glucose uptake by the mammary gland, while the insulin responsiveness of adipose tissue and muscle are decreased. Insulin also induces the expression of milk protein genes, and IGF-1 stimulates cell division leading up to lactogenesis. The importance of the lactogenic complex of the hormones insulin, prolactin and glucocorticoids has been demonstrated using *in vitro* culture of bovine mammary tissue. The specific hormones that are involved in regulating lactogenesis vary among different species.

Progesterone inhibits the initiation of lactogenesis but has no effect on milk yield once lactation is established. Progesterone has been suggested to work by increasing the mammary threshold to prolactin, by altering the secretion of prolactin from the pituitary or acting as a glucocorticoid receptor antagonist. Levels of progesterone are high during

gestation and serve to inhibit lactogenesis until just before parturition. The level decreases about 2 days before parturition to remove the inhibition of milk synthesis. High levels of progesterone do not inhibit milk synthesis in pregnant lactating cows, due to low levels of progesterone receptors in mammary tissue.

ST does not appear to be involved in the onset of lactation in cattle. ST was not lactogenic when added to slices of mammary tissue cultured *in vitro* or when given to late pregnant cattle in the dry period.

Lactation can be induced hormonally in non-pregnant cattle that have been dry for at least 30 days. They are injected for 7 days with high levels of oestrogen plus progesterone, followed by increasing prolactin and, finally, treatment with glucocorticoids. After 3 weeks the cows are milked, since milk removal is important in stimulating mammary growth and inducing lactation. This method is not effective in all cows and the level of milk production is lower than if the cow gave birth and started lactation naturally. Milking different quarters of the udder at different times prepartum changes the composition of the milk from each quarter at parturition. This demonstrates that local autocrine/paracrine factors within each quarter of the gland also affect the final activation of milk secretion.

Maintenance of lactation (galactopoiesis)

Hormonal effects

Thyroid hormones are required for maximal milk production. During lactation there is decreased conversion of T_4 to the active hormone T_3 in liver and kidney but increased conversion to T_3 in the mammary gland (see Section 3.6). This enhances the priority of the mammary gland for metabolites compared with other body tissues. Surgical thyroidectomy or treatment with radioactive iodine, which is sequestered in the thyroid gland to destroy thyroid function, decreases milk yield. Administration of thyroid hormones or iodinated casein (thyroprotein) causes a temporary increase in milk production. However, milk yield after treatment with iodinated casein is below normal, so there is no net benefit.

High levels of exogenous glucocorticoids and oestrogen decrease milk production in an established lactation. Physiological levels of glucocorticoids stimulate milk production in rats but the results are ambiguous in ruminants. There is no change in CBG during lactation in cattle and corticoids are not limiting to milk yield.

Prolactin is required for the maintenance of milk production in rats and rabbits, with decreases in milk yield of 50% or more after bromocriptine administration. Bromocriptine administration is much less effective in ruminants, except for the lactating ewe. Suckling induces a threefold release of prolactin over 30 min after milking, which is much less dramatic than the peripartum prolactin surge associated with lactogenesis. Milking is thought to increase prolactin secretion by decreasing prolactin-inhibiting factor (PIF) from the hypothalamus. There is a correlation between milk yield and prolactin in blood 5 min after milking, but milk yield and prolactin levels before or 1 h after milking were not correlated.

Prolactin levels increase with increasing temperature and photoperiod. Prolactin levels are also affected by stress, so blood samples must be taken carefully. Increasing photoperiod from 8 to 16 h day^{-1} increased milk yield by 6–10% in cattle. Increasing photoperiod also stimulated release of prolactin and IGF-1, but the galactopoietic effect of these hormones has not been proven.

Cows can be pregnant and lactating at the same time. Administration of bovine placental lactogen increases milk yield but little endogenous placental lactogen is normally released into the maternal circulation, so it will usually have a nominal effect.

Milk removal

Milk removal from the mammary gland is necessary for the maintenance of lactation. Milk secretion rate is less affected by long milking intervals in high-producing dairy cows than in low-producing cows. Increasing milking frequency from two to three or four times per day can increase milk yield to a similar extent as treatment with bST (see below). The rate of milk secretion increases only in the part of the gland that is milked more frequently. Since all sides of the udder are exposed to the same systemic hormones, this suggests that local autocrine factors within the individual parts of the mammary gland affect milk production.

The increased milk production is not simply due to manipulation of the gland but requires actual

removal of the milk. A 7.6 kDa milk whey protein isolated from goat's milk has been proposed as a feedback inhibitor of lactation (FIL). This protein would act in an autocrine manner to reduce the rate of milk secretion, stimulate the degradation of newly synthesized casein and reduce prolactin receptor numbers on mammary epithelial cells. However, more research is needed to establish FIL in regulating milk production. TGFβ, insulin-like growth factor binding protein-5 (IGFBP-5) and lactoferrin levels are increased during apoptosis of mammary epithelial cells and may be responsible for cessation of milk production. TGFβ represses casein synthesis and secretion and IGFBP-5 antagonizes IGF-1 to decrease cell proliferation, while lactoferrin has antimicrobial and immunomodulatory effects.

Acute accumulation of milk in the mammary gland also increases intramammary pressure, which activates sympathetic nerves to decrease mammary blood flow and limit the supply of hormones and nutrients to the gland. Mechanical milking is less effective than suckling in milk removal. This is probably due to decreased oxytocin production, which would decrease the ejection of the milk from the alveoli and small milk ducts into the large ducts and cistern of the udder. This can be corrected by injection of oxytocin, although high doses of oxytocin can cause desensitization of the udder. For more information, see Weaver and Hernandez (2016).

Effect of bST

The effects of ST on growth and carcass composition are discussed in Section 3.4. ST is not galactopoietic in rats but it is the major galactopoietic hormone in cattle. High-producing dairy cows have high levels of ST and low levels of insulin. There is a large increase in circulating ST early in lactation, which mobilizes fat reserves, inhibits lipogenesis and blocks insulin-dependent glucose uptake in non-mammary tissues. This is followed by a second period, where ST is at a lower concentration. There is also a decrease in the number of ST receptors in the liver around calving time, which decreases the production of IGF-1 and reduces the negative feedback on ST early in lactation.

The development of recombinant DNA technology made possible large-scale production of bovine somatotrophin (bST) for use in improving the efficiency of milk production in dairy cattle. Recombinant bST was approved for use in dairy cattle prior to 1990 in the former Soviet Union, Brazil, Mexico, South Africa, Bulgaria and Czechoslovakia and in the USA in 1994, but it is not approved in Canada. It has also been shown that bST increases growth rate in lambs and to increase milk production in ewes and buffalo.

Milk yield increases by 10% when bST is administered in early to mid-lactation and by 40% in late lactation. The percentage increase depends on the dose, formulation, nutrition programme, herd health and, most importantly, management and environmental factors. Similar increases in milk yield are found in all breeds. Various effects of parity have been reported. It may be more cost effective to administer bST after peak lactation, when the effect of treatment is greatest. Treated cows become more persistent in milk production, so milk yield decreases at a slower rate than in untreated cows after peak production (Fig. 4.6). This also extends the calving interval and results in fewer calves being born per year. Increasing ST by treatment with GHRH from the anterior pituitary is as galactopoietic in dairy cattle as treatment with ST directly.

During the early transition phase of bST treatment, there is a delay of a few weeks in increasing the voluntary feed intake, although milk yield increases immediately. This puts the cow in negative energy balance, so body fat stores are mobilized and body condition scores are reduced. There may be a slight increase in the milk fat content during this time and a slight decrease in milk protein, due to a limited supply of amino acids. Adequate body condition score should be achieved prior to calving.

Cows treated in the long term with bST increase their nutrient intake by an average of 1.5 kg day^{-1} to support the increased milk production. The absolute nutrient requirements for maintenance and the nutrient requirements per unit of milk produced by the dairy cow are not changed by bST treatment. However, the increased milk production results in an overall increase in feed efficiency, since the requirements for maintenance are a smaller percentage of the overall requirements. Cows treated with bST are similar to genetically superior cows at the same level of milk production and should be fed according to the level of milk production. Genetic selection for increased milk production may have resulted in increased endogenous levels of ST.

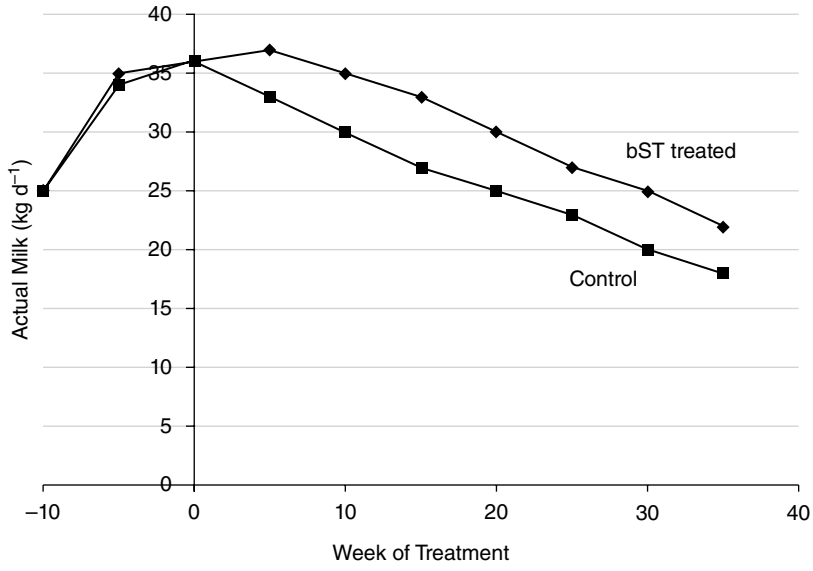

Fig. 4.6. Effect of bST on milk production.

For interest

An amino acid polymorphism (F279Y) in the transmembrane domain of the growth hormone receptor affects milk yield and composition. However, the allele that is associated with increased milk yield may also have negative effects on reproduction (Oikonomou *et al*., 2008).

Inadequate nutrition will decrease the response to bST. Using more grain can increase the energy density of the ration, but dietary buffers are then needed to maintain pH balance in the rumen. Feeding ruminally inert fat, such as the calcium salts of long-chain fatty acids (Megalac®), also increases the dietary energy. Sufficient levels of high-quality rumen-undegradable protein are also needed. The diets needed by bST-treated cows are more expensive but the income from milk production is increased over feed costs. For more information on the effects of bST on nutrient requirements of dairy cattle, see NRC (1994). For more information on the effects of bST on reproduction and health, see the articles by Dohoo *et al.* (2003a, b).

Mechanism of action

ST does not bind to receptors in the bovine mammary gland but acts by partitioning additional nutrients to the mammary gland during lactation for the increased synthesis of lactose, protein and fat. Insulin normally increases the utilization of acetate by adipose tissue for lipid synthesis and thus is involved in partitioning nutrients away from milk synthesis and towards body tissues. Levels of insulin in blood are negatively correlated with milk yield. Adipocytes become less sensitive to insulin, especially during early lactation, and ST acts on the adipocytes to make more energy from fat available to the mammary gland. Activities of acetyl-CoA carboxylase and fatty acid synthase are decreased in adipose tissue by ST treatment. This increases the level of non-esterified fatty acids (NEFAs) in plasma, which can be oxidized by peripheral tissues or used for the synthesis of milk fat. Insulin also encourages glucose sparing from peripheral tissues and increases the glucose available for milk production by the mammary gland. The lipolytic effects of bST are most prominent during the first two-thirds of long-term treatment during lactation.

ST also stimulates secretion of IGF-1 from the liver, which increases proliferation and survival of mammary cells. IGF-1 infusion into mammary glands is not galactopoietic, so these mitogenic responses of IGF-1 may not be important in the galactopoietic effects of ST. However, IGF-1 may compete with insulin for binding to the insulin

receptor in peripheral tissues, thus contributing to the insulin resistance caused by ST. There are at least six binding proteins for IGF-1, which either inactivate or enhance the activity of IGF-1 or have biological activities of their own. These proteins are present in the blood as well as in the interstitial spaces of the mammary gland.

Delivery

The Monsanto Corporation developed and marketed bST in 1994, using the trade name Posilac® and this was acquired by Eli Lilly (Elanco) in 2008. Recombinant-produced bST has up to eight amino acids added to the amino terminus, depending on the manufacturing method. This has resulted in four different recombinant bST products that differ from the terminal Ala residue: Somagrebove® (Met-Asp-Gln; American Home Products), Somavubove® (Ala; Pharmacia and Upjohn), Sometribove® (Met; Elanco) and Somidibove® (Met-Phe-Pro-Leu-Asp-Asp-Asp-Asp-Lys; Elanco).

In solution, bST undergoes deamidation, with a tenfold increase in deamidation from 5°C to 37°C. Covalent cross-links also form between bST molecules and result in hydrophobic aggregates that precipitate at neutral pH and high protein concentrations. This can cause extremely slow and incomplete release of bST from a sustained-release formulation. Aggregation can be reduced somewhat using detergents such as Tween 20 or hydroxypropyl-β-cyclodextrin and sorbitol, but the high protein concentrations needed for prolonged release of more than 1 month are still a problem.

Two major commercial preparations are Posilac® (500 mg Sometribove® Met-bST zinc suspension; Elanco) and Boostin® (alanyl bST in vehicle composed of 1200 mg vitamin E acetate and 300 mg of lecithin; LG Life Science, Seoul, Korea). They are prolonged-release formulations injected subcutaneously every 2 weeks starting 57–70 days after calving and continuing until the end of lactation. Suspensions of bST in oil have been made for sustained release and to stabilize the molecule by excluding water. Aluminium monostearate has been used as a dehydrating or gelling agent with bST dispersed in oil at 10–50% by weight. Microparticles of bST have also been made using waxes, stearates, fatty acid anhydrides, sucrose, sodium sulfate, polysorbate 80 or sodium benzoate and dispersed in a carrier vehicle. Once injected, the microparticles are dissolved and the bST is released over time (see Section 2.4). Implants have been made of compressed pellets of bST powder, with excipients such as polyanhydride, polycaprolactone, polyesters, cholesterol and ethylcellulose to slow the release of bST. Implants can suffer from problems of incomplete release of bST from their interior, the necessity of sterilizing them and problems with placing them. For more information, see Raux *et al.* (2022).

Safety concerns of bST use

Milk composition is only slightly altered by bST by altering the nutrient requirements of the cow. bST increases milk production and also the problems normally associated with high milk production, which may be a result of the increased milk yield rather than a direct effect of bST. Treatment with bST increased the risk of clinical mastitis by 25% and increased the risk of a cow failing to conceive by approximately 40%. There is also an estimated 55% increase in the risk of developing clinical lameness in bST-treated cows. This might be due to negative effects of bST on connective tissue and bone development (see Section 3.4).

Cows treated with bST can take longer to come into oestrus after parturition than untreated cows, particularly if their body condition is not adequate. Adequate nutrition should be provided to replenish body condition during late lactation or during the dry period. There is no effect on the number of services required per cow once the animal starts cycling, and levels of gonadotrophin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are unchanged by bST treatment. Delaying bST treatment until after peak lactation or until conception has occurred can counteract the negative effects on reproduction in dairy cows. For the report of the Expert Panel on the effects of bST in dairy cattle, see the papers by Dohoo *et al.* (2003a, b).

It is thought that bST has no oral activity in adult humans and is destroyed by digestion; however, young infants can absorb intact proteins. Bovine IGF-1 has the same sequence as human IGF-1, but levels in milk are only increased by abnormally high doses of bST and are not above those found in human breast milk. Both bST and IGF-1 will be at least partially denatured by heat during pasteurization. The Food and Drug Administration of the USA reviewed more than 120 studies prior to approval of bST but it was not

approved for use in Canada, due to the potential negative effects on animal health and not because of concerns about human health.

Factors affecting milk composition

Milk is composed of three main components: proteins, lipids and oligosaccharides (mainly lactose), along with vitamins, nucleic acids, cells and other bioactive compounds. The genetics and nutrition of dairy cows affect the composition of milk. There are large differences in milk fat content among different breeds of dairy cows, ranging from 5.1% for Jersey to 3.7% for Holstein cows. Likewise, Jerseys produce milk with 3.8% protein compared with 3.1% protein for milk from Holstein cows. Milk fat and protein contents increase in later stages of lactation, and frequency of feeding and method of feeding also affect milk composition. Sows have higher milk fat content compared with most other mammals. Undoubtedly, hormones are involved in the underlying mechanisms for these differences in milk composition.

For interest

A polymorphism (B) in the whey protein β-lactoglobulin gene decreases its expression and increases the yield of cheese per unit of milk. The BB kappa casein genotype is associated with high milk protein content and improved rennet coagulation properties during cheese-making.

Reasons for manipulating the composition of milk include: (i) improving the processing of milk and manufacture of milk products; (ii) improving the nutritional quality of milk; and (iii) using milk as a source of nutraceuticals. All components of milk can be altered to some degree. The amount of fat and the fatty acid composition of milk fat are most easily altered and can be changed over a range of 3 percentage units. Protein content can be changed over 0.5 percentage units, while the lactose content of milk cannot be changed, except under extreme dietary manipulation. Lactose is the most important osmotic component of milk, so changes in lactose synthesis are accompanied by changes in water volume and thus milk yield. Holstein cows synthesize more lactose relative to protein and fat and have more dilute milk than Jerseys. Lowering the metabolic demand for glucose to synthesize lactose may be beneficial, especially in early lactation.

Milk protein

Milk proteins play a crucial role in the processing properties of milk, such as solubility, water bonding, heat stability, renneting and foaming. Milk proteins are also a source of bioactive components in milk. The nitrogen fractions of milk are casein (mainly αs-casein (αs1 and αs2), β-casein, and κ-casein, 78%), whey (β-lactoglobulin, α-lactalbumin, lactoferrin, immunoglobulins, serum albumin, glycomacropeptides, enzymes and growth factors, 17%) and non-protein nitrogen (5%). Milk protein content can be increased by 0.4 percentage units if the forage proportion of the diet is reduced to 10% of the total dry matter, but this is less than the minimum required to avoid digestive problems. The increase in milk protein is due to increased production of propionate and microbial protein when increased levels of rapidly fermentable carbohydrate are fed. Increases in dietary protein only have modest effects on milk protein levels, due to poor capture of amino acids by the mammary gland. Increases in dietary fat cause a decrease in milk protein content, due to decreased extraction of amino acids by the mammary gland. Infusion of glucose along with increasing insulin in blood will increase the protein concentration in milk and this may provide a method to manipulate the composition of milk.

The primary purpose of milk proteins is to provide a source of readily digestible proteins with a balanced amino acid content for the young. However, the digestion of milk proteins produces a wide array of bioactive peptides that modulate digestive and metabolic processes (Fig 4.7). This includes peptides with effects on nutrient uptake (phosphopeptides, casomorphins), immune function (immunopeptides, casokinins, casomorphins) and neuroendocrine function (casokinins). Peptides with antimicrobial properties that are active against bacteria, yeast and fungi are produced by proteolysis of lactoferrin and casein. Anti-hypertensive peptides that inhibit the angiotensin-converting enzyme to reduce blood pressure are derived from casein. Anti-thrombotic peptides that inhibit platelet function are derived from casein and lactotransferrin. Casein phosphopeptides form a complex with calcium ions and provide a passive means for increased calcium absorption in the small intestine

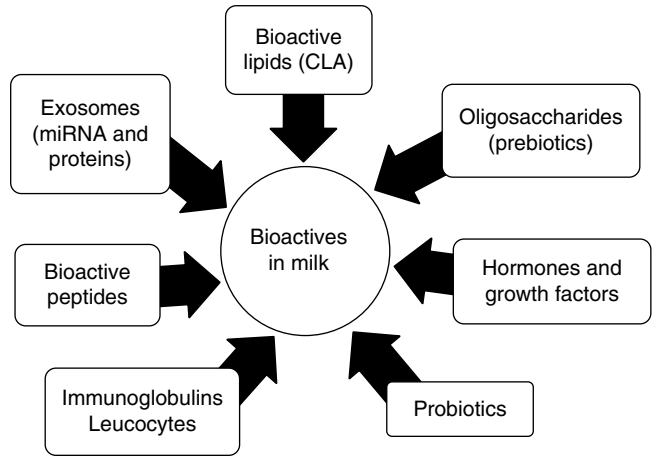

Fig. 4.7. Bioactive compounds in milk.

as well as recalcification of dental enamel. Immunomodulatory peptides that affect the immune system and the cell proliferation response are derived from casein. Casomorphins are opioid peptides derived from casein. They are absorbed from the intestine to produce analgesic effects and induce sleep. They also inhibit intestinal peristalsis and motility, modulate amino acid transport and stimulate the secretion of insulin and somatostatin. Thus, there is a lot of potential for producing functional ingredients and high value-added products from milk. Many of these proteins could be salvaged from by-products of the manufacture of dairy products. For more information, see the reviews by Vargas-Bello-Pérez *et al*. (2019) and Lin *et al*. (2021).

Milk fat

Fat is present in milk in the form of milk fat globules, which are composed of a triglyceride core covered with a triple membrane comprising phospholipids and other polar lipids and proteins derived from mammary gland cellular membranes. The components of the milk fat globule membrane have positive effects on development, gut functionality, metabolism and cognition. The size of the milk fat globule ranges over 3 orders of magnitude, from less than 200 nm to over 15 μm, depending on the lipid and protein composition of the membrane, and this affects the chemical and sensory characteristics of dairy products. The membrane content is higher for smaller globules, due to the higher ratio of surface area to volume of the globule, so the smaller globules will have increased content of membrane-derived bioactive compounds.

The largest proportion of milk lipids is in the form of triglycerides, along with lesser amounts of diglycerides and monoglycerides. The fatty acid composition of milk can be altered to produce a more desirable product. For example, increasing the content of C18:0, and particularly C18:1, and decreasing the content of C14:0 and C16:0 may help to reduce plasma cholesterol and would result in a softer butter. Conjugated linoleic acid (CLA) occurs predominantly in meat and dairy products. It has been shown to reduce the growth of many types of tumours and to reduce the risk of artherosclerosis (see Section 3.9). Altering the fatty acid profile of milk has also been reported to lower plasma cholesterol in people consuming the product. Increasing the fat content has traditionally been a focus in dairy breeding programmes but altering milk composition through breeding strategies is effective only in the long term.

Volatile fatty acids produced in the rumen, especially acetate, are the major precursors for synthesis of milk fat. Acetate production is increased with some high-fibre diets, while other fibres increase the production of propionate, which is a precursor for glucose and lactose synthesis. Milk fat content is decreased as the proportion of concentrate is increased in dairy rations, particularly in late lactation. This is due to increased production of *trans*-10 CLA isomers by ruminal microorganisms. A severe

depression in milk fat content is found after infusion of *trans*-10, *cis*-12 CLA but not *cis*-9, *trans*-11 CLA.

Milk fatty acids are derived from dietary long-chain fatty acids (50%), from microbial synthesis or from body stores of fat (5–10%), or are directly synthesized from acetate and β-hydroxybutyrate by the mammary epithelial cells (40–45%). The amount of milk fatty acids from *de novo* synthesis in the mammary gland is higher in early lactation. Microbial action in the rumen produces branched-chain and odd-carbon-number fatty acids. Dietary unsaturated fatty acids are biohydrogenated to saturated fatty acids by ruminal microorganisms, so unsaturated fatty acids must be ruminally protected to be deposited in milk. Rumen-protected fat sources include whole oilseeds, amides of fatty acids, calcium salts of fatty acids and formaldehyde-treated fats.

Hormones affect the delivery of nutrients to the mammary gland and the activities of the acetyl-CoA carboxylase and fatty acid synthase enzymes involved in fat synthesis in the mammary gland. The mammary gland cannot produce fatty acids longer than 16 carbons. The Δ9-desaturase enzyme in mammary secretory cells converts saturated stearic acid from ruminal biohydrogenation to oleic acid, which is secreted in milk. This enzyme is also responsible for conversion of *trans*-11 vaccenic acid (*trans*-11-C18:1) from the rumen to *cis*-9, *trans*-11 CLA, which is the major isomer of CLA in milk. Feeding fresh pasture or supplementing diets with low levels of fish oil or sunflower oil increases levels of *cis*-9, *trans*-11 CLA in milk.

The balance between fatty acids synthesized in the mammary gland and those available from the diet or microbial synthesis can be altered by dietary means. Feeding cows palm-derived feed supplements rich in palmitic acid (C16:0) can increase milk fat by 0.2–0.4 percentage points, depending on the ration formulation. Feeding sources of unsaturated fatty acids, such as sunflower and safflower supplements, increases the content of unsaturated fatty acids, particularly C18:1, and lowers the content of C16:0 in milk. High levels of unsaturated fatty acids are toxic to the rumen microflora and this is controlled by converting the fatty acids in feed supplements into their calcium salts. Levels of eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) in cow's milk can be increased to the levels found in human breast milk by including a fishmeal-containing supplement in the diet of dairy cattle. For information on modifying milk fat composition, see Kliem and Shingfield (2016).

Other bioactive compounds in milk

Mammary secretions (milk and colostrum) serve both as a source of nutrients (lipids, carbohydrates and proteins) and as a source of bioactive compounds that provide protection and signalling molecules to promote growth and development in the young. Early colostrum feeding has been shown to dramatically affect the development of the reproductive tract and endometrial and uterine gene expression in nursing piglets and to ultimately improve the reproductive success of the animals. These studies led to the lactocrine hypothesis – the concept that biologically active agents (e.g. growth factors, hormones and bioactive peptides) in colostrum and milk act to programme postnatal development. Aside from bioactive peptides, milk contains exosomes, oligosaccharides, bacteria and hormones that modulate digestive and metabolic processes.

Exosomes are nanosized endosome-derived membrane vesicles (40–100 nm in diameter) found in milk. They contain mRNA, microRNA (miRNA), DNA, proteins and lipids that are involved in immunity, proliferation and cellular signalling and can transfer these bioactive components to neonatal animals. miRNAs are small regulatory RNA molecules of 19–24 nucleotides in length that play important roles in a wide range of physiological and pathological processes. Milk miRNAs and proteins in exosomes are stable to intestinal digestion and permeable to the intestinal barrier. Colostrum has a higher amount of immune-related miRNAs and proteins in exosomes than mature milk, which can be transferred to neonates to regulate the immune system and inflammatory response and stimulate gastric/pancreatic digestion. Several abundant exosomal milk miRNAs are conserved in sequence homology and are present in milk from humans and pigs, which may indicate that they are evolutionarily selected to benefit the newborn.

Milk oligosaccharides have anti-infective and prebiotic functions in the neonate. They are in highest concentration in colostrum at 1–2 g l^{-1}, gradually decreasing during early lactation to 100 mg l^{-1} in mature milk. Oligosaccharides can be fermented in the large intestine into short-chain fatty acids, including butyric acid, which regulate mucosal immunity, gut integrity and colonic health. Oligosaccharides selectively stimulate growth of beneficial probiotic bacteria such as *Bifidobacterium* and *Bacteroides* and can also prevent pathogens from adhering to the mucosa, since they contain

analogues to various receptors for microbes (see Section 3.11). Probiotic lactic acid bacteria are also present in milk and they might originate from the maternal intestine, translocate to milk through the bloodstream and subsequently colonize the gastrointestinal tract of neonates.

The teat surface can also contain a high diversity of bacteria from the environment.

Traditionally, immunoglobulins in colostrum were considered to be the only source of adaptive immunity from the sow to piglets. However, leucocytes (neutrophils, macrophages and lymphocytes) in sow's milk can also be a source of immunity. IGF-I, IGF-II, insulin, relaxin and EGF are present in both sow colostrum and milk, although the concentrations of these hormones are relatively low in milk compared with those in blood. For more information, see the reviews by Zhang *et al*. (2018), Lin *et al*. (2021) and Thum *et al*. (2021).

Metabolic diseases related to lactation

The transition period from pregnancy to lactation is a critical time period for dairy cows. After calving, the cow's metabolic demands shift from supporting the growing calf to producing colostrum and milk to feed the newborn. High-producing dairy cows have increased nutritional requirements to support milk production. However, feed intake typically decreases in the transition period, so there are insufficient nutrients to support the demands of milk production. This requires that body stores of fat and muscle be mobilized to compensate for the decreased availability of energy and nutrients and the cow is in negative energy balance (NEB). Mobilization of fat stores can result in ketosis. There can also be insufficient supplies of calcium, resulting in hypocalcaemia and milk fever. Cows that succumb to metabolic or infectious disease after calving are at greater risk of reduced milk production and reproductive performance and increased risk of other diseases. This decreases both the profitability for the dairy producer and the welfare for the cow.

Ketosis

Ketosis results from a high demand for glucose for milk synthesis at a time when the cow is in NEB (Fig. 4.8). This usually occurs in early lactation, when the cow may not consume sufficient feed to produce enough propionate for glucose synthesis. This leads to the catabolism of protein from muscle and mobilization of fat from adipose tissue, which increase levels of non-esterified fatty acids in plasma. As the capacity of the liver to metabolize fatty acids becomes limiting, levels of ketone bodies

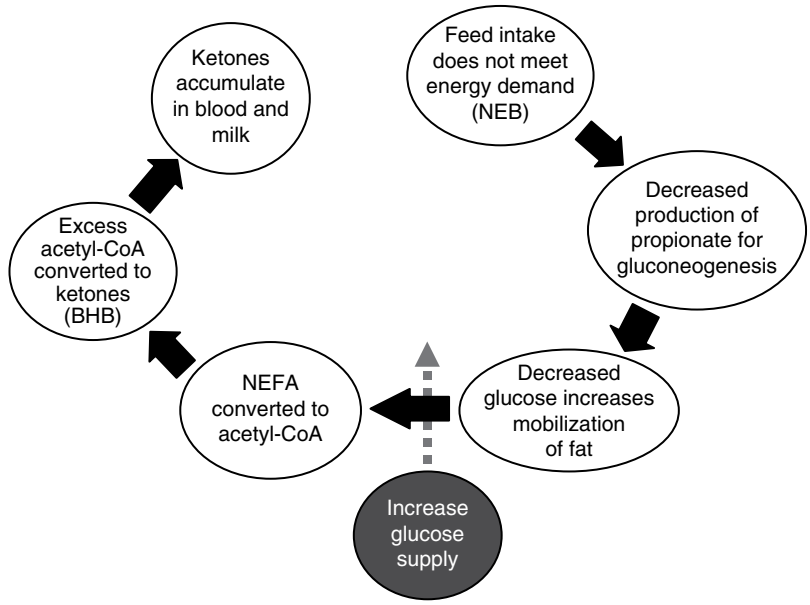

Fig. 4.8. Causes and treatment of ketosis.

(β-hydroxybutyrate (BHB), acetone and acetoacetate) increase in the circulation. Ketone bodies can be used by muscle as an alternative fuel source to glucose, sparing glucose for milk production, but can accumulate to cause hyperketonaemia, acetone breath and ketonuria, and unmetabolized fat can accumulate in the liver. Ketosis is diagnosed by measuring BHB in blood or milk samples. The threshold for subclinical ketosis (without outward physical signs) is blood BHB > 1.1 to 1.4 mmol l^{-1}. Cows with clinical ketosis typically have blood BHB > 2.5 mmol l^{-1}, with decreased milk production, decreased feed intake, dry manure, rapid loss of body condition and neurological signs in severe cases. Accurate, practical and economical cow-side tests are available to measure BHB in blood, milk, or urine. Concentrations of BHB in milk reflect concentration in blood but are only 10–15% as large.

Ketosis is an extremely common disease in lactating dairy cattle, with a worldwide prevalence of 20% and a lactational incidence of 40%. The prevalence of ketosis is generally higher in Jersey cows (19%, ranging from 11.4% to 25%) than in Holstein cows (14%, ranging from 0 to 28%). The pathogenesis of ketosis as a metabolic disease is multifactorial, and many tissues and metabolic pathways are altered in the ketotic cow. There are also differences in ketosis susceptibility between cows in their first lactation compared with subsequent lactations, and ketosis is more prevalent during 5–21 days in milk compared with 22–45 days in milk. Ketosis also increases the risk of other diseases (including metritis, displaced abomasum and severity of mastitis), depresses appetite, milk yield and body weight, impairs reproductive performance and increases the likelihood of premature culling. The cost of ketosis is approximately Can$300 per cow, costing the Canadian dairy industry nearly Can$115 million annually.

An imbalance in the insulin/glucagon ratio, abnormal liver function, adrenal corticosteroids, thyroxine and mineral and vitamin deficiencies have all been implicated in the etiology of ketosis. Moderate levels of body fat prepartum and increasing feeding rapidly postpartum are desirable, but appetite and intake are normally depressed postpartum.

Treatments for ketosis are aimed at increasing body glucose (Fig. 4.8). Administration of cortisol or ACTH can raise blood glucose but may be detrimental in the long term. Traditional treatment regimens have included B vitamins, insulin and intravenous dextrose infusion, preferably in combination with oral propylene glycol, which is converted to propionate and used in gluconeogenesis. Of these, oral administration of 300 ml propylene glycol once daily for 3–5 days is the recommended treatment. However, ketosis treatments to date still result in nearly 50% of cows either remaining chronically affected or relapsing. The reason why NEB develops into hyperketonaemia and subsequently ketosis in some but not all cows, and why some cows respond to treatment while others do not, have not yet been completely defined. For more information, see Gross and Bruckmaier (2019).

Milk fever

Milk fever (parturient paresis) from hypocalcaemia occurs at the onset of lactation and has been recognized in cattle for over 200 years. Hypocalcaemia can reduce the productive life of a dairy cow, making it 3–5 times more likely to develop postpartum disease and 50% more likely to be removed from the herd in early lactation than healthy cows. It is also a serious animal welfare concern in dairy farming. Subclinical hypocalcaemia can affect up to 50% of postpartum cows and is an economically important metabolic condition, while clinical milk fever affects less than 5% of postpartum cows. Clinical symptoms of milk fever include reduced appetite and rumen motility, inhibition of urination and defecation, cessation of milk production, lateral recumbency and eventual coma and death if left untreated. Cows recovering from milk fever have a greater incidence of rumen dysfunction, left-sided abomasal displacement and ketosis than cows that have never had milk fever. The hypocalcaemia of milk fever also decreases immune function, leading to increased incidence of mastitis. Cows suffering from milk fever also had dystocia, retained placenta, displaced placenta and uterine prolapse and have an abnormally long interval between calving and their first postpartum ovulation.

Milk fever is due to a severe drop in blood calcium, which disrupts neuromuscular function. The concentration of calcium is 12 times higher in milk than in blood, with calcium in milk bound to both casein micelles and ionized forms. A cow producing 10 l of colostrum requires about 23 g of Ca, which is about nine times the total plasma pool of calcium, while a cow in early lactation milking 40 l day^{-1}

requires 56 g of calcium. Calcium in plasma is derived from the diet and from bone storage reserves. At parturition, the cow must bring more than 30 g of calcium per day into the plasma pool. However, it takes about 14 days to condition the effective release of calcium from bone and the early postpartum cow may not receive enough dietary calcium, due to a depressed appetite. This can result in low blood calcium (hypocalcaemia) of less than 6.5 mg 100 ml^{-1}, compared with 10 mg 100 ml^{-1} for normal blood calcium, and can cause milk fever.

HORMONES INVOLVED Blood calcium levels are increased by the coordinated actions of the calcitrophic hormones parathyroid hormone (PTH) and 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$) and decreased by calcitonin (Fig. 4.9). PTH release from the parathyroid gland is under tonic inhibition in response to high blood calcium, which is detected by the G protein-coupled calcium-sensing receptor (CaSR). The CaSR is expressed in several tissues involved in calcium homeostasis, including the parathyroid gland, kidney, gut, bone and mammary gland. When levels of calcium in the blood decrease, PTH is released to increase calcium reabsorption from the glomerular filtrate in the kidney and calcium resorption from bone osteoclasts. The total soluble calcium in bone fluids of the typical cow is estimated at 6–10 g and this is increased by an additional 6–8 g during metabolic acidosis. During lactation, parathyroid hormone-related protein (PTHrP) produced by the mammary gland activates osteoclasts and stimulates the rapid

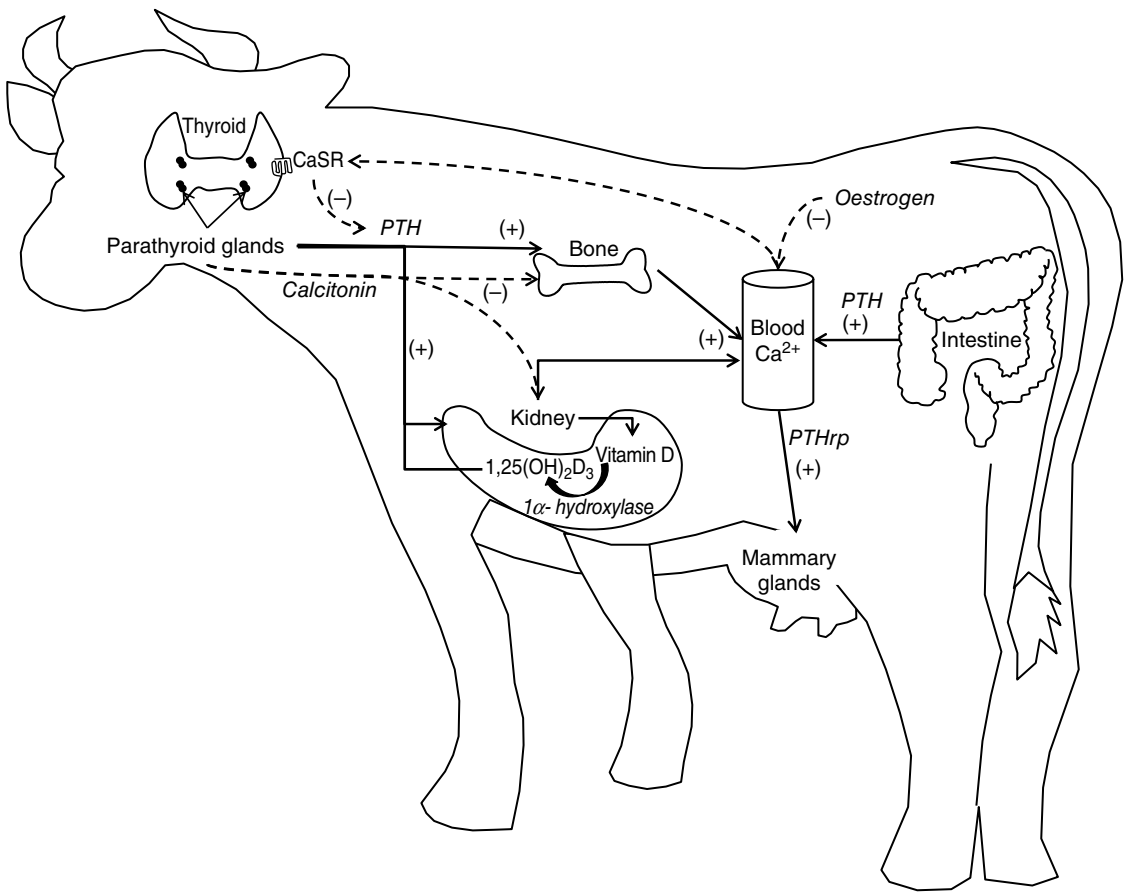

Fig. 4.9. Hormonal regulation of calcium metabolism.

transport of the calcium from the bone fluids to the extracellular fluid.

PTHrP produced locally by the mammary gland epithelial cells is thought to be involved in stimulating calcium transport from blood to milk. CaSR on the basolateral side of the mammary epithelium regulates calcium homeostasis during lactation to control the amount of calcium in the milk and blood. Ionized Ca^{2+} is a critical intracellular second messenger and thus it is buffered in the cytoplasm by binding to proteins, or by storage in the Golgi apparatus by the secretory plasma Ca^{2+} ATPases (SPCA1/2) and in the endoplasmic reticulum by the sarco(endo)plasmic reticulum ATPase (SERCA). During late pregnancy, activity and expression of calcium transporters, pumps and modulators are increased to move Ca from the blood into the milk. Transport of calcium across the basolateral membrane into the mammary gland occurs through a Ca^{2+} influx channel (Orai1), while calcium is actively pumped into milk by the plasma membrane Ca^{2+} ATPase 2 (PMCA2).

PTH also induces the 1α-hydroxylase enzyme in the kidney, which activates vitamin D to $1,25(OH)_2D_3$. The $1,25(OH)_2D_3$ stimulates the synthesis of the calcium transport protein transient receptor potential vanilloid channel type 6 (TRPV6) and the cytosolic protein calbindin-D9K that move calcium across the intestinal epithelial cells and the magnesium-dependent Ca-ATPase that pumps calcium out of the cell into the plasma. In the distal tubule of the kidney, uptake of calcium occurs via transient receptor potential vanilloid channel type 5 (TRPV5) where it binds to calbindin-D28K and is exported to the plasma by an active sodium–calcium exchanger type 1 (NCX1). The $1,25(OH)_2D_3$ also acts synergistically with PTH to increase the release of calcium from bone and increase the renal reabsorption of calcium. $1,25(OH)_2D_3$ also stimulates the differentiation of bone marrow stem cells into osteoclasts. Levels of $1,25(OH)_2D_3$ are decreased by stimulating the expression of fibroblast growth factor 23 (FGF23) and the 24-hydroxylase (CYP24A1) which inactivates both $25(OH)D_3$ and $1,25(OH)_2D_3$.

Calcitonin released from the parafollicular C-cells of the thyroid increases calcium deposition in bone by inhibiting the effects of PTH. Calcitonin also stimulates the excretion of calcium and phosphorus in the kidney. Oestrogen (produced by the placenta) also decreases the resorption of calcium from bone. Serotonin increases mammary PTHrP production to increase calcium transport into the milk, causing bone resorption.

PREDISPOSING FACTORS Cold stress in subzero temperatures can induce hypocalcaemia. Milk fever is more prevalent in over-fat cows with fatty liver than in thin cows, as mobilization of fatty acids can affect blood calcium levels.

Some breeds of cows, especially Swedish Red-and-White and Jersey, are more susceptible to milk fever. Lower numbers of intestinal receptors for $1,25(OH)_2D_3$ are present in Jerseys than in age-matched Holsteins. The decreased receptor number would limit the effectiveness of $1,25(OH)_2D_3$ in stimulating the reabsorption of calcium. Periparturient ewes are also affected by milk fever.

The incidence of milk fever increases dramatically in third and later lactations. Older cows have increased milk production and greater demands for calcium. However, the ability to resorb calcium from bone and transport calcium across the intestinal epithelial cells, as well as the activity of $1,25(OH)_2D_3$, decrease with age. This is due to a decrease in the intestinal receptors for $1,25(OH)_2D_3$ and an increase in the activity of the 24-hydroxylase enzyme that inactivates $1,25(OH)_2D_3$.

TREATMENT AND PREVENTION Treatment of milk fever consists of supplementing blood calcium levels until the bone and intestinal transport systems adapt to provide the necessary calcium. Intravenous infusion of 23% calcium borogluconate is most widely used but this can result in high plasma calcium if administered too quickly, causing cardiac arrest, so subcutaneous injection can be used. There can also be a rebound hypocalcaemia around 24 h after administration of intravenous calcium, due to decreased PTH. About 25% of cows treated this way require additional treatment. Calcium and phosphorus supplements can also be given around parturition, in the form of calcium carbonate, calcium chloride gels or dicalcium phosphate. A paste of calcium propionate has been used to give a more sustained calcium release and to provide propionate as a gluconeogenic precursor.

Stimulating calcium turnover from bone before calving can reduce the incidence of milk fever. This is done by feeding limiting amounts of dietary calcium (100 g day^{-1}, or 70 g day^{-1} for small breeds) for 2 weeks before calving. Insufficient dietary magnesium reduces calcium mobilization from bone, while excess dietary phosphorus can interfere

with vitamin D metabolism and cause milk fever. Dry-cow rations need to be balanced for calcium (0.39%), phosphorus (0.24%) and magnesium (0.23%). A low-calcium diet can be achieved by feeding a calcium binder, zeolite A, a synthetic sodium–aluminum silicate which binds calcium, phosphorus and magnesium in the rumen. Cows fed zeolite A for 2–3 weeks prepartum have increased serum calcium concentrations during the immediate peripartum period.

Injection of vitamin D_3 or its synthetic analogues within 8 days prepartum may also be effective in preventing milk fever. However, cows treated with vitamin D compounds, including 1α-hydroxyvitamin D_3, develop hypercalcaemia and are unable to produce endogenous $1,25(OH)_2D_3$. More active and longer-lasting vitamin D analogues, such as $24\text{-F-}1,25(OH)_2D_3$, have been developed as an experimental implant.

PTH infusions or injections can prevent milk fever, with about 20 times more PTH needed for intramuscular than intravenous treatment. A sustained release of PTH via an implant has been suggested.

Anionic salts such as ammonium chloride and magnesium sulfate also stimulate the release of calcium from bone. These salts are rather unpalatable and must be mixed with feed in two daily feedings and not fed for more than 3–4 weeks. Hydrochloric acid that has been diluted and mixed with molasses can also be used as a source of dietary anions, but HCl is dangerous to handle and corrosive to machinery. The principle of feeding strong dietary anions is to create a negative dietary cation–anion difference (DCAD) which causes a compensated metabolic acidosis. Adding anions reduces the DCAD to reduce the pH of the blood and urine. The DCAD is calculated as:

$$\text{DCAD} = (0.38Ca^{2+} + 0.3Mg^{2+} + Na^{+} + K^{+}) - (Cl^{-} + 0.6SO_4^{2-})$$

This takes into account the average absorption of the different ions in cattle.

A DCAD of –50 to –100 mEq kg^{-1} of diet or urinary pH of between 5.5 and 6.2 has been suggested as optimal for prepartum diets. Mild acidosis increases tissue responsiveness to PTH, resulting in increased resorption of calcium from bone and increased synthesis of $1,25(OH)_2D_3$. This may be due to increased expression and ligand affinity of PTH receptors at lower pH.

An alternative approach to increasing dietary anions is to lower the levels of dietary cations. This is particularly important if the DCAD of the diet is greater than 250 mEq kg^{-1}, as it will be difficult to add enough anions to lower the DCAD to –100 mEq kg^{-1} without seriously affecting the palatability of the feed. Potassium is the cation present in highest amounts in ruminant forages, and growing conditions that produce consistently low levels of potassium in forages would be beneficial in reducing the cation content of the feed.

The strategies for preventing milk fever can be summarized as follows. If the dietary DCAD is below 250 mEq kg^{-1} of feed, anions can be added to the diet to produce mild acidosis and increase the sensitivity of the PTH receptors. If the DCAD is above 250 mEq kg^{-1} feed, calcium gels can be administered to increase the passive absorption of calcium. PTH, vitamin D analogues or low-calcium diets can also be given to increase the activity of the calcitrophic hormones and increase the available calcium. For more information, see Wilkens *et al.* (2019).

4.2 Egg Production

Key concepts

- In female birds, the left ovary matures and the right ovary regresses, while the male develops two functional testes. The asymmetric development of the gonads is controlled by *PITX2*.
- The cortex of the left gonad forms an ovary in the presence of oestrogen, while in males the medullas of both gonads form testes, since oestrogen is not present.
- High expression of Z-linked *DMRT1* drives testis development in ZZ males.
- There is a hierarchy of follicular development where growing follicles progress gradually towards maturity.
- FSH is involved in maintaining the hierarchy of size in the developing follicles and in the selection of the preovulatory follicle.
- Ovulation occurs due to an LH surge that is driven by progesterone from the largest follicle.
- The age-related declines in egg production and in shell quality can be addressed by induced moulting.
- Conventional induced moulting involves the withdrawal of feed and water and a reduced lighting schedule to shut down the reproductive system and reduce BW.

- Alternative methods for inducing moulting include feeding high-fibre and low-energy diets or diets low in Ca and Na or high in Zn.
- The eggshell provides protection and allows gas exchange for the embryo.
- Shell strength is determined by the mineralization process, which involves both shell-matrix proteins and calcium salt crystals.
- The organic matrix directs the nucleation and controls the growth of crystals in the mineral layer and has a role in antimicrobial defence.
- The calcium used for shell formation comes from the diet and is reversibly stored in medullary bone.
- Ca transport in the intestine is regulated by vitamin D, while both PTH and vitamin D regulate Ca storage in bone. Ca deposition in the shell gland is driven by carbonic anhydrase.

Sexual development and gonadogenesis

Female chickens have a unique reproductive structure, since they have only one functional ovary. During embryonic development, birds start out with two undifferentiated gonads. As the male matures, it develops two functional testes, while in the female the left ovary matures and the right ovary regresses. The physiological basis for only one ovary developing is not understood but may be related to weight constraints for flight and the restraints imposed by the additional nutrient requirements of the organ. However, these factors are not important in laying hens. Interestingly, some species of wild birds, particularly hawks, develop two functional ovaries and oviducts. A line of Rhode Island Red chickens with two oviducts has also been described (Wentworth and Bitgood, 1988). This suggests that it may be possible for two functional ovaries and oviducts to develop in chickens. This has the potential to increase egg production, although the physiological and metabolic constraints of producing twice as many eggs may make this impossible. More likely, egg production may increase slightly and might be more prolonged, with ovulation alternating between the two ovaries.

Producing birds of the desired phenotypic sex is also important, since the broiler industry prefers males, due to their improved growth rate and improved lean yield, while the layer industry uses only females. Millions of chicks are therefore sexed as day-old chicks using sex-linked feather growth or colour identification or by manual vent sexing, and male layer-strain birds are culled because they do not produce eggs and are not commercially viable to grow out for meat. This practice has long been recognized as a serious animal welfare concern and a waste of animal resources. Various hormonal and physical manipulations can somewhat alter the sexual phenotype and produce pseudo sex-reversed birds, but no reliable method has yet been found to permanently alter the phenotypic sex of commercial poultry.

Reliable and economical methods for sexing birds *in ovo* would allow for incubation of eggs that only carry the embryo of the desired sex. A sample can be taken from the egg and assayed for sex-specific markers, or different types of spectroscopy may be used to differentiate eggs that carry either male or female embryos. Another approach is to tag the Z chromosome of females with a marker gene, such as green fluorescent protein (GFP). Crossing these females with a normal ZZ male would result in all the male offspring receiving this tag, which could be used to sex chicks *in ovo* at the point of lay. For more information, see Doran *et al*. (2017).

Hormonal effects

During embryonic development, the primordial germ cells (PGCs) settle in the urogenital ridge region of the embryo after around 3 days of incubation. At 5–7 days of incubation, the gonads are bipotential and can form either ovaries or testes. The undifferentiated gonad is composed of an inner medulla, which is potentially testicular tissue that produces androgens, and an outer cortex, which is potentially ovarian tissue that produces oestrogens. The medulla contains the primary sex cords, with germ cells that will form seminiferous tubules in males. In females, the cortex of the left ovary gives rise to secondary sex cords that contain germ cells. Sex differentiation and regression of the right ovary in chickens begins at 6.5 days of embryo development. The mechanism for asymmetric development of the gonads is summarized in Fig. 4.10. In developing genetic females, the gonads produce mostly oestrogens, while androgens predominate in the genetic male. The major hormone present then affects the pattern of gonadal development, with oestrogen playing a key role in both gonadal development and sexual differentiation of the brain to a female pattern of behaviour. Steroid

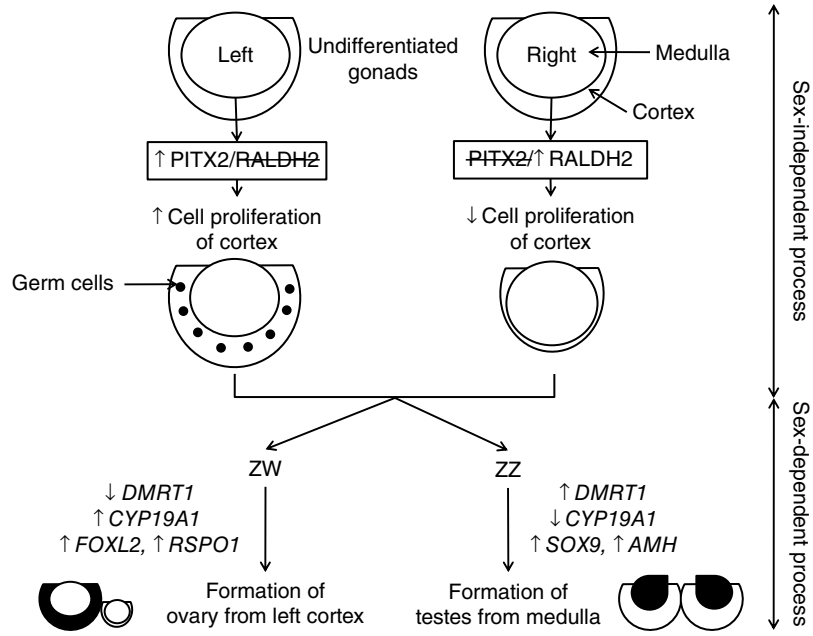

Fig. 4.10. Mechanism of asymmetric gonad development. The events shown in the sex-independent process lead to asymmetric development of the cortex in both sexes. The sex-dependent processes leading to formation of an ovary or testes are driven by oestrogen.

hormones also play a role in the development of secondary sex characteristics as the birds mature. Androgens stimulate comb growth, spur growth, male feathering and copulatory behaviour, while oestrogens cause female feathering patterns and inhibit male phenotypic and behavioural expression.

However, cells in the body of the chicken have an innate sexual phenotype, and sexually dimorphic gene expression can occur in embryos prior to gonadal sex differentiation. Pre-gonadal mesodermal tissue that was transplanted into male embryos before gonadal differentiation developed as ovarian tissue. This demonstrates that autonomous sexual development occurs by direct genetic effects within the cells, a process known as cell autonomous sex identity (CASI). Sexual differentiation thus involves both direct genetic effects and indirect hormonal mechanisms.

Gonad development in fish and reptiles can be altered by treatment with exogenous sex steroids; androgens induce the formation of functional testes, while oestrogens induce the formation of functional ovaries. The sensitivity of fish and reptiles to exogenous hormones is the basis of several assays for endocrine disruptor chemicals (EDCs) (see Section 6.4). Mammalian gonads are insensitive to exogenous hormone treatment, while the gonads of birds are intermediate in response. Treatment with an aromatase inhibitor prevents the synthesis of oestrogen and causes the development of a functional male phenotype, although the female growth pattern is not altered. Removal of the left ovary or natural regression of the left gonad after infections or tumours induces the right gonad to develop as a semi-functioning testis and a male phenotype to develop. Castrated males continue to grow like males, while genetically male embryos treated with oestrogen at 4 days of age develop a transient female phenotype.

The aromatase enzyme is expressed in the medullas of the left and right gonads of female embryos but not in male embryos. The oestrogen receptor (ERα) is expressed in the left gonad and not the right gonad of both sexes, but the expression in males is restricted to a very early stage of development. The lack of oestrogen receptor in the right gonad could explain why the right gonad does not develop as an ovary. The oestrogen receptor is transiently present in the left male gonad, which explains why the left gonad can be sex reversed in the male by administration of oestrogen. The effect of oestrogen on sexual differentiation in the brain

is mediated by ERβ. The oestrogen receptor does not normally play a role in the male, since the aromatase gene is not expressed, and oestrogen levels would normally be low.

Genetic effects

In birds, the female is the heterogametic sex (ZW) and the male is homogametic (ZZ), while in mammals the male is the heterogametic sex (XY) and the female is homogametic (XX). The mammalian Y and avian W chromosomes are both small and contain very few genes. In mammals, female is the 'default sex', and the *SRY* gene on the Y chromosome is the master sex determinant, directing the undifferentiated gonad to develop into a testis (see Section 5.1). In birds, the *SRY* gene is absent and testis development is driven by the doublesex and Mab-3-related transcription factor 1 gene (*DMRT1*), which is expressed in the genital ridge and Müllerian ducts after 3–4 days of incubation. It is located on the Z chromosome and is therefore expressed in higher levels in ZZ (male) embryos than in ZW (female) embryos. The development of the avian testis is dependent on increased expression of *DMRT1*, which decreases production of oestrogen by aromatase (*CYP19A1*) and activates sex determining gene on y (SRY) box 9 gene (*SOX9*) and anti-Müllerian hormone gene *AMH* to stimulate development of testis cells. Lower expression of *DMRT1* by having only one copy of the gene in female gonads activates the ovarian pathway genes, *FOXL2* and *CYP19A1*. Most downstream genes involved in sex differentiation are conserved among vertebrates. The transcription factor gene *SOX9* and the hormone gene *AMH* are up-regulated in developing testes and the signalling factor genes *WNT4* and forkhead transcription factor-2 (*FOXL2*) are up-regulated in developing ovaries (see Section 5.1).

The pituitary homeobox 2 gene (*PITX2*) is expressed only in the left gonad of both sexes and is responsible for asymmetric development of the gonads (Fig. 4.10). Artificial expression of *PITX2* on the right-side gonad in females prevents its regression and allows the cortex to develop and produce germ cells. The expression of *PITX2* in the cortex of the left gonad suppresses the expression of retinaldehyde dehydrogenase (*RALDH2*), which decreases the production of retinoic acid (RA) and thereby decreases signalling due to binding of RA to the nuclear receptors RAR and RXR. This increases the expression of the receptors ERα and SF-1. SF-1 stimulates the expression of cyclin D1 to increase cell proliferation in the cortex. The lack of *PITX2* expression in the right gonad leads to increased RA signalling and suppression of cell proliferation due to decreased SF-1 and cyclin D expression.

Both Müllerian and Wolffian ducts are present during early development before sexual differentiation has occurred. When an ovary is present and the synthesis of oestrogen occurs, the Müllerian ducts develop into oviducts, uterus and cloaca, and the Wolffian ducts regress. Oestrogen production in females by CYP19A1 is increased by FOXL2 and oestrogen stimulates the left cortex to form an ovary. Oestrogen increases expression of R-spondin 1 (RSPO1), a regulator of the WNT/b-catenin signalling pathway (see Section 5.1), and represses the expression of the AMH receptor, AMHRII, which protects the left Müllerian duct from regression.

In males, the presence of a testis directs the Wolffian ducts to develop into vasa deferentia, and Müllerian ducts to regress. The medulla develops in both testes, while the cortex develops only on the left side, as it does in females. The medullas of both gonads form testes, since oestrogen is not present. Addition of aromatase into a developing 10-day-old male embryo decreases the expression of testes-determining genes (*DMRT1*, *SOX9* and *AMH*) and results in the development of ovarian tissue and up-regulation of ovarian development genes *FOXL2* and *RSPO1*. The development of the Müllerian ducts is inhibited by AMH, which is produced by the developing testis. AMH in chicken has been cloned and is a 644 amino acid glycoprotein that is a member of the TGFβ family. It may also inhibit aromatase activity. The action of AMH is inhibited by oestrogen, and the lack of oestrogen receptors in the right ovary results in regression of the right Müllerian duct. AMH may be controlled by *SOX9* as well as other genes encoding transcriptional factors involved in sex determination and sexual differentiation, including *DAX1*, *WT1*, *SF1*, *PITX2* and *DMRT1* (see Section 5.1).

DAX1 is expressed at similar levels in both male and female gonads during differentiation and may be more important in regulation of steroidogenesis. *WT1* is also expressed at similar levels in male and female gonads. *SF1* is expressed by day 3.5 of incubation and, in contrast to mammals, is higher in

female than in male gonads, with expression up-regulated in developing ovaries after the onset of differentiation.

Manipulating the pattern of gene expression during sexual differentiation may lead to methods for reliably altering the phenotypic sex of poultry or preventing the regression of the right ovary in females. For more information on sexual differentiation in birds, see Major and Smith (2016), Hirst *et al.* (2018) and Estermann *et al.* (2020).

Regulation of follicular development and egg production

Commercial chickens (pullets) begin to lay eggs as they approach sexual maturity at 18–20 weeks of age. However, not all hens begin to lay eggs at the same time. Egg production increases to a maximum of about 90% of hens producing an egg every day over about 2 months, as all the hens come into lay, and then gradually decreases as the hens get older. Eggs are laid in clutches, with a laying hen in the first half of her egg-laying cycle having a clutch of 50 eggs laid in succession without a pause. An ideal production curve would be when maximum egg production is reached immediately and then maintained indefinitely (Fig. 4.11).

In high-producing egg-laying hens, there is a well-organized and continuous hierarchy of follicular development, where growing follicles progress gradually towards maturity with little atresia of developing follicles. Follicular development is under the control of the pituitary gonadotrophins but the precise mechanisms are not well understood in birds. Follicles are composed of a central oocyte surrounded by two layers of endocrine cells, an inner layer of granulosa cells and an outer layer of thecal cells. Multiple follicles begin to mature at the same time and are classified as primordial follicles (smaller than 0.08 mm in diameter), primary follicles (from 0.08 mm up to 1 mm in diameter), undifferentiated pre-hierarchical follicles (from 1 mm up to 8 mm in diameter) and preovulatory/hierarchical follicles (larger than 9 mm in diameter). Follicles that grow to 10 mm in diameter are recruited to the stage of accumulating yellow yolk; granulosa cells begin to proliferate and theca gradually forms outside the basement membrane. Between four and eight yellow growing follicles are present at any time and these are arranged in a hierarchy of size, based on their stage of maturation. One dominant (F1) follicle is recruited from this group on approximately a daily basis to be the preovulatory follicle that progresses to ovulation.

Yolk lipoproteins are synthesized in the liver and delivered via the circulation to the theca layer of the developing follicle. They then cross the basement membrane and pass between the granulosa cells to access the oocyte, where they are taken up

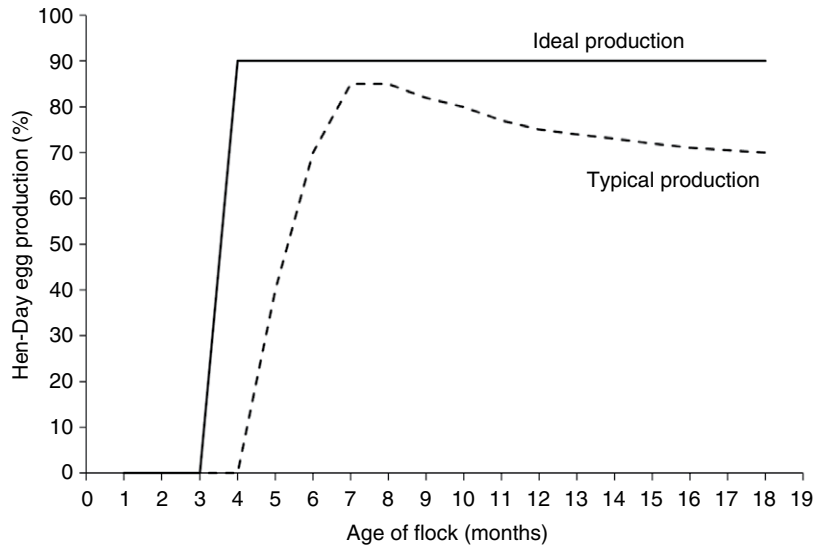

Fig. 4.11. Changes in egg production of laying hens with age.

via a specific lipoprotein receptor with 8 ligand-binding domains (LR8) located on the oocyte membrane. Follicular growth and yolk deposition have been studied by feeding birds gelatin capsules containing fat-soluble dyes, such as Sudan black or Sudan red. As the yolk is deposited in the growing follicle, the dye forms a coloured ring. This can be seen by slicing the yolk after the egg has been hard-boiled.

Before recruitment as yellow follicles, the layers of the theca cells in the numerous small follicles produce dehydroepiandrosterone (DHEA), androstenedione and oestrogens (Fig. 4.12). Following recruitment, the yolk-filled follicles produce progesterone and decreased quantities of oestrogens. As the follicles continue to mature, they lose the ability to produce androgens and oestrogens, and the granulosa layer of the largest preovulatory follicle produces large quantities of progesterone. This surge of progesterone stimulates the release of LH, which acts by positive feedback to increase progesterone production by the largest follicle, which ultimately results in ovulation. Plasma levels of LH and progesterone peak at 4–6 h prior to ovulation. LH stimulates steroidogenesis in all follicles, with androgens and oestrogens produced by the theca cells of small follicles. Following ovulation, the postovulatory follicle undergoes apoptosis and is degraded by several matrix metalloproteinases.

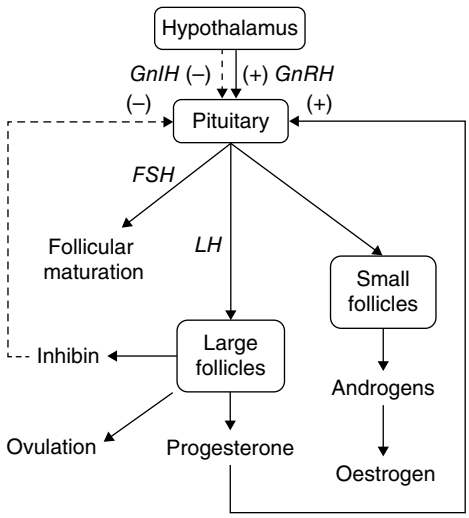

Fig. 4.12. Regulation of follicular growth and maturation.

Ovulation is stimulated by a surge in LH concentrations and the timing of ovulation is controlled by the circadian rhythm, which is normally set by the light–dark cycle (see Section 1.4). In the absence of a light–dark cycle, eggs are laid at all times throughout the day, although some hens may establish a circadian rhythm from other environmental cues. As little as 1.25 h of darkness is sufficient to establish the circadian rhythm from the photoperiod. The transition from light to dark is important and eggs are laid 12–18 h after darkness begins.

The length of the ovulatory cycle and follicular maturation can range from 23 h to 28 h in different birds. However, hens in normal production are kept on a light–dark cycle of 14L–10D, totalling 24 h, which fixes the period of time that LH is released and ovulation can occur. Birds that take longer than 24 h to mature a follicle will therefore not always have a follicle ready for ovulation when levels of LH have peaked. This results in a non-egg-laying day or a pause in the sequence or 'clutch' of eggs that are laid. If hens are kept on a 28-h-day cycle, virtually all hens will be able to mature a follicle in this period and one egg will be laid for each light–dark cycle. A bird that has a rate of follicular development that is less than 24 h will not ovulate the mature follicle until the LH surge. Selection for birds with rapid rates of lay using short 22 h photo-schedules has been used to increase the rate of egg production, but lower egg weights were obtained. In chickens with low egg-laying rates, such as broiler breeders that are fed *ad libitum*, follicular development is not as well organized and ovulations are irregular or often multiple. This can be managed by limiting feed intake, which establishes a hierarchy of follicular development and improves egg production. For more information on ovary and follicular development in hens, see Johnson (2012, 2014).

High levels of prolactin (PRL) stimulate broodiness behaviour in poultry and decrease LH, which decreases progesterone and oestradiol production by the ovary and the number of developing follicles. Immunization against prolactin in broody hens restores LH levels and decreases broodiness (see Section 6.1). In non-broody chickens, immunization against PRL slows down ovarian follicular development and reduces egg-laying performance. This suggests that, at lower levels, PRL stimulates the development of the follicular hierarchy and improves egg laying in poultry. For more information, see Hu *et al.* (2017).

Gonadotrophin release by the pituitary is stimulated by GnRH and inhibited by gonadotrophin-inhibitory hormone (GnIH), produced by the hypothalamus. GnRH and GnIH act directly on pituitary gonadotropes via specific G protein-coupled receptors (GnRHR and GnIHR). There are two forms of GnRH in the chicken (Table 4.2), with GnRH-I being the most prevalent. Immunization against GnRH-I but not GnRH-II inhibits ovulation. Mammalian GnRH is also active in chickens.

GnIH in chicken is a dodecapeptide (SIKPSAYLPLRFamide) and is a member of the RFamide family of peptides that can act as neurotransmitters and neuromodulators. Melatonin from the pineal gland acts directly on GnIH neurons via the melatonin receptor to induce GnIH expression. The terminals of GnIH neurons are localized in the median eminence and in the preoptic area (POA) of the hypothalamus, along with GnRH neurons. GnIH acts on GnRH neurons in the POA to inhibit GnRH release. Another RFamide neuropeptide, kisspeptin, has a stimulatory effect on GnRH neurons, leading to the release of GnRH (see Section 5.1).

As the hen ages, there is decreased gonadotrophin production from the anterior pituitary gland in response to GnRH. This results in a reduced number of follicles reaching the final stages of maturity, so a mature follicle is not always available for ovulation and the rate of lay decreases. However, since fewer follicles are developing, they receive a proportionally greater amount of yolk, causing an increase in egg size.

FSH is involved in maintaining the hierarchy of size in the developing follicles and the selection of the preovulatory follicle. FSH induces granulosa cell proliferation, differentiation and follicle selection by stimulating the expression of FSH receptors, steroidogenic acute regulatory protein (StAR) and cytochrome P45011A1 (CYP11A1). Levels of FSH are decreased by inhibin, which is produced by the four largest preovulatory follicles (F1–F4), in particular the largest F1 follicle (Fig. 4.12). Inhibin is a gonadal glycoprotein that is a member of the TGFβ family of peptides (see Section 5.1). It comprises two subunits, α and βA or βB, to produce inhibin A (αβA) and inhibin B (αβB). Activin is a related protein that comprises two β subunits to form activin A (βAβA), activin B (βBβB) and activin AB (βAβB) and has opposite physiological effects on FSH. Inhibin reduces the secretion of FSH from the anterior pituitary gland without affecting LH production. It has also been shown to have paracrine effects in the ovary, acting as a competitive FSH receptor antagonist in mammals. Follistatin is a soluble protein that binds the β subunits of activin to inhibit it; it also binds inhibin with less affinity but its physiological role with inhibin is not completely understood. The IGFs also have important effects on the control of reproductive function in the ovary. They appear to act as paracrine/autocrine regulators of follicular growth and differentiation. For more information, see the review by Onagbesan *et al.* (2009).

Application

The expression of inhibin in the F1–F4 follicles and plasma levels of inhibin are higher in hens that lay at a low rate compared with those that lay at a high rate. The α subunit of inhibin has been cloned and sequenced and this information has been used to develop inhibin conjugates for immunization of hens. Immunization against inhibin increased the number of follicles that were recruited into the preovulatory hierarchy in chickens, and advanced the onset of lay and increased the rate of egg production in Japanese quail and chickens.

Manipulation of moulting

Towards the end of the laying cycle, there is a normal age-related decline in egg production and quality, and particularly in shell quality, so that egg production becomes uneconomic for the producer. Rather than replace the flock, some producers will

Table 4.2. Structure of mammalian GnRH, chicken GnRH-I and chicken GnRH-II.

	Amino acid number									
	1	2	3	4	5	6	7	8	9	10
mGnRH	Pyro-Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
cGnRH I	Pyro-Glu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
cGnRH II	Pyro-Glu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2

induce a moult to initiate new follicular growth and an additional period of egg production, thereby reducing bird-replacement costs and improving profitability. During the moult, egg laying stops, the reproductive tract involutes and feathers are shed. Conventional induced moulting involves the withdrawal of feed or feeding altered diets for about 2 weeks and restricted access to water at the beginning of the moult. The lighting schedule is also changed from 16 h of light to 8 h or less. Upon completion of the moult, birds are returned to a normal layer diet and lighting schedule, new feathers develop, the reproductive system is rejuvenated and egg production resumes, with improved shell strength, egg quality and production rates.

Moulting rejuvenates the reproductive system but is associated with a 25–35% loss of body weight and reduction of body fat, with the goal of returning the flock to their juvenile pullet body weight at the start of their second laying cycle. The loss of body weight and feathers raises concerns for the welfare of hens because of increased bird distress and mortality, particularly if feed withdrawal is used to induce the moult. Feed withdrawal also reduces the ability of the hen to resist colonization of the gut by pathogenic bacteria, increases the risk of salmonella infection and lowers the immune response. As a result, alternative methods have been investigated and codes of practice in many countries now specify the use of non-feed-withdrawal methods to induce moulting. This involves reduction of daily energy intake through restriction of nutrients while providing constant access to low-nutrient-density feed and modification of light exposure.

The practice of induced moulting extends the productive lives of hens that would normally be culled when they reach a low level of egg production or shell quality. Replacing older flocks with new pullets instead of moulting would increase the number of breeder flocks, breeder farms, hatcheries and pullet-rearing farms. It would also increase the number of male chicks that would have to be destroyed and generate more spent hens, which would have to be removed.

Endocrine effects

Natural moulting in the jungle fowl (*Gallus gallus*), the wild progenitor of the laying hen, occurs with the onset of broodiness (see Section 6.1). The jungle fowl hen consumes very little food and water while incubating the eggs and loses approximately 20% of her body weight. Prolactin is involved in the development of broodiness, and a high level of prolactin reduces the release of GnRH from the hypothalamus and LH from the pituitary; it may also directly inhibit ovarian steroidogenesis. In species that exhibit broodiness, the levels of prolactin increase when moulting begins, but this does not occur in commercial laying hens that are induced to moult by fasting. However, treatment of laying hens with prolactin can induce moulting.

During fasting-induced moulting, the level of corticosterone increases to mobilize body energy reserves. Smaller increases in corticosterone occur when methods that take longer to induce moulting are used. The increased level of corticosteroids inhibits GnRH stimulation of LH release and there is a reduced response of the ovary to LH-induced production of progesterone. This results in regression of the oviduct and gonadal atrophy; it also reduces the cell-mediated immune response, with an increase in the heterophil:lymphocyte ratio.

Thyroid hormones are also involved in moulting (see Section 3.6). Levels of thyroid hormone increase and subsequently ovarian activity decreases. The decreased release of oestrogen removes the suppression of activity of the feather papillae, which are activated by thyroid hormone and progesterone to form an underlying new feather, which expels the existing feather.

Applications

Alternative methods to feed removal for inducing moulting have been investigated. Feeding high-fibre and low-energy and low-protein diets has been shown to be effective in inducing moulting, reducing body weight and maintaining the normal gut microflora. Lucerne can be used, due to its higher crude protein and calcium content and the slow transit time through the digestive tract, resulting in better digestion and microbial fermentation. Feeding diets low in sodium or calcium or high in zinc is as effective as feed withdrawal to induce moulting. Feeding a calcium-deficient diet results in decreased plasma LH, decreased egg production and ovary regression by 6–9 days.

Hormone-induced moulting methods include using GnRH agonists to reduce GnRH release (see Section 3.3), injection of progesterone or melengestrol acetate (see Section 3.2) and treatment with thyroid hormone or thyroactive proteins

(see Section 3.6). However, the intensive hen handling, higher costs and variable production performance after hormone-induced moulting make these methods less practical for use by the poultry industry. For more information, see Patwardhan and King (2011) and Glatz and Tilbrook (2021).

Eggshell formation

Poor eggshell quality, resulting in cracked shells, is a major source of economic losses to the layer industry, and eggs with shell defects can account for 6–10% of all produced eggs. Poor shell quality occurs due to environmental factors, such as temperature and stress, as well as nutritional factors, such as the availability of dietary calcium and levels of minerals such as phosphorus and chloride. Older hens metabolize calcium in feed less efficiently than younger birds and poor shell quality, along with decreased egg production, is a major cause of culling in older hens. The eggshell is also important for successful development of the chick embryo, as it provides protection from damage, infection and desiccation and provides a source of calcium for skeletal development. The development of a thicker shell is undesirable, since this would reduce the exchange of gas and water and make it more difficult for the embryo to hatch. Rather, it is the mineralization process that involves both shell-matrix proteins and calcium salt crystals that affects shell strength, so that a mineral layer of about 0.3 mm thickness can withstand a static pressure of more than 3 kg. The shells of eggs at the end of a hen's laying cycle can still contain the same amount of calcium but eggshell strength can be decreased due to poor structural organization within the mineral layer.

After ovulation, the ovum is fertilized in the infundibulum and the perivitelline membrane is formed. Albumen is added during the 3–4 h that the ovum and yolk travel down the magnum. Albumin contains proteins such as ovotransferrin and lysozyme, which reduce the growth of microorganisms, and ovalbumin to stabilize the developing embryo. The shell membranes are added further along in the isthmus (Fig. 4.13) and these are a fibrous structure that forms the innermost layers of the eggshell. The inner layer of the shell membrane is not calcified while organic aggregates known as mammillary knobs are deposited on the surface of the outer shell membrane to act as mineralization sites for calcium carbonate. The egg then enters the shell gland or uterus, where fluid is pumped into the ovalbumin (egg white), causing it to swell or 'plump' to its final size so that the outer eggshell membranes have good contact with the uterine mucosa. About 6 g of calcium carbonate (calcite) and a thin layer of calcium phosphate (apatite) are laid down in the 18 h while the egg slowly rotates in the shell gland; this is one of the most rapid biomineralization processes known. The ionic and protein constituents of the uterine fluid that direct shell calcification change progressively during the stages of eggshell formation of initiation (5 h),

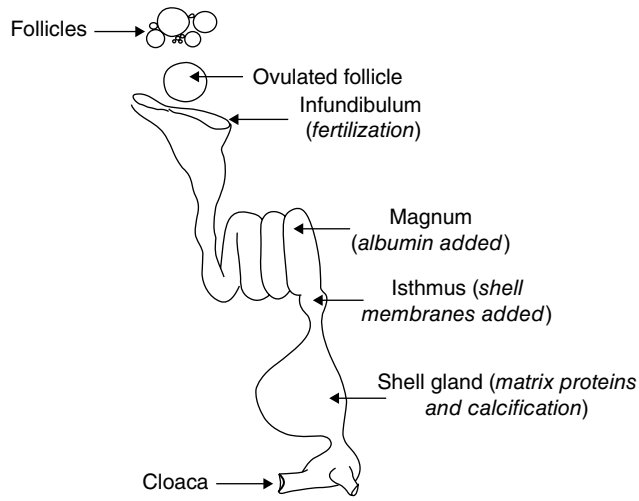

Fig. 4.13. The reproductive tract of poultry.

growth and rapid calcification (12 h) and termination of mineralization (2 h), which suggests that these proteins play specific roles during the calcification process.

The eggshell consists of an organic matrix (3.5%), water (1.5%) and mineral layers (95%) and accounts for 10% of the total egg weight. The organic matrix constituents control the mineralization process and these have been studied with the goal of improving eggshell quality, since it is the structural organization within the mineral layer that is the most important factor affecting shell strength. The eggshell is divided into six layers (Fig. 4.14). The innermost layers are the membranes that enclose the yolk and albumen. The outer membrane is anchored to the first calcified (cone) layer through mammillary knobs that are distributed on the surface of the membrane. This is followed by the palisade layer, which consists of an array of crystals arranged perpendicular to the shell surface that makes up two-thirds of the eggshell mineral thickness, and then a vertical layer of crystals that are of a higher density than the palisade layer. An organic cuticle covers the outer surface of the eggshell and contains the majority of shell pigments. The cuticle is thought to control water exchange, play a role in temperature control and in preventing microbial penetration and serve as camouflage and possibly parental recognition of the egg. Pores span the eggshell to allow for gas and water exchange for the developing embryo.

Shell matrix

The organic matrix is composed of a mixture of proteins and polysaccharides that directs the nucleation and controls the growth of crystals in the mineral layer, thus affecting the mechanical strength of the shell. In addition, some matrix proteins have a role in antimicrobial defence. Some of the proteins involved in regulating mineralization are acidic and are located at the interface between the crystal and the matrix, while others are found within the crystals. Crystal growth is initiated by deposition of calcium carbonate on organic aggregates that are present in the mammillary layer on the outer surface of the eggshell membranes. Crystal structure is affected by protein–mineral interactions and competition for space from crystals growing from adjacent centres of nucleation. The shell membranes are necessary for shell deposition and affect the pattern of mineral growth.

For interest

Replacement of part of the shell of a snail by eggshell membranes resulted in an avian pattern of mineral formation in the snail shell. This is due to the specific protein patterns on the eggshell membrane that regulate mineralization.

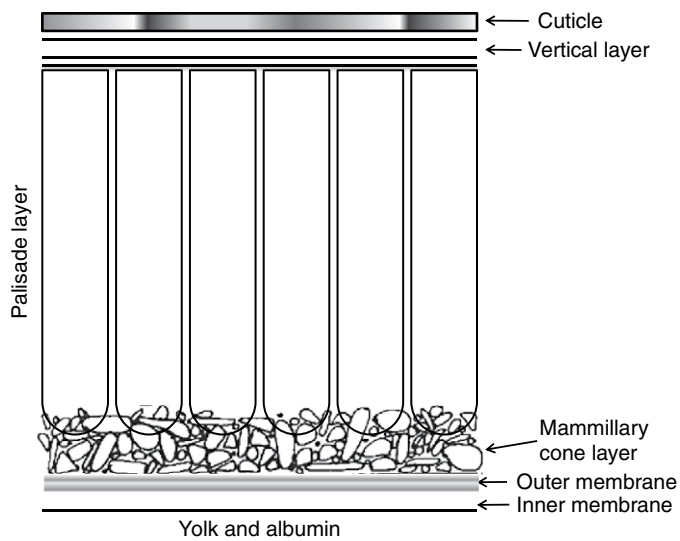

Fig. 4.14. Diagram of the various layers in the shell.

Eggshell proteins are progressively secreted into the uterine fluid at different concentrations, depending on the stage of shell calcification, while levels of calcium and bicarbonate remain high. These include: (i) 'egg white' proteins: ovalbumin, the most abundant egg white protein; lysozyme, an antimicrobial protein; and ovotransferrin, which sequesters iron necessary for bacterial growth; (ii) ubiquitous proteins that are found in many tissues, including osteopontin (OPN, a bone-matrix phosphorylated glycoprotein) and clusterin, a widely distributed secretory glycoprotein; and (iii) eggshell-specific matrix proteins: ovocleidins (OCs) and ovocalyxins (OCXs). The eggshell matrix also contains proteoglycans, primarily keratan sulfate and dermatan sulfate. These macromolecules influence the organization of crystal growth by controlling the size, shape and orientation of calcite crystals. Changes in shell-matrix proteins have also been shown during the growth of shells from molluscs and echinoderms. Polymorphisms in ovalbumin and ovotransferrin (*OVT*) genes have been associated with crystal size, while polymorphisms in ovocleidin-116 and ovocalyxin-32 genes were associated with crystal orientation.

OPN is expressed in the shell gland and secreted into the lumen only during the period of shell calcification; it is concentrated in the mammillary layer and outer shell membrane. OPN synthesis is induced by the mechanical strain of the egg in the shell gland and it is not expressed if the egg is removed prematurely from the gland. Ovocleidin-17 (OC-17) is an abundant shell-matrix protein that is concentrated in the mammillary layer. Aside from its effect on calcite crystal growth, it inhibits the growth of Gram-positive bacteria. Ovocleidin-116 (OC-116) is widely distributed throughout the palisade layer. Ovocalyxin-32 (OCX-32) is localized in the outer palisade layer and is involved in the terminal phase of eggshell formation. It may also provide antimicrobial protection for the egg. OCX-36 is found in the inner part of the shell near the shell membranes. It is part of a family of proteins that initiate the inflammatory host response when a pathogen is detected.

For interest

Single nucleotide polymorphisms (SNPs) in the *OC-116* gene are associated with eggshell thickness, elastic modulus and eggshell shape; an SNP in *OCX-32* is associated with mammary layer thickness; an SNP in *OPN* is associated with fracture toughness; and an SNP in the ovalbumin gene is associated with breaking strength and shell thickness (Dunn *et al*., 2012).

Functional studies of shell-matrix proteins have involved studying the effects of these proteins on delaying calcium carbonate precipitation and affecting the size, shape and orientation of crystals formed *in vitro* from a supersaturated salt solution. Uterine fluid collected during the growth phase enhances calcium carbonate precipitation, while soluble extracts of eggshells, or uterine fluid collected towards the completion of the shell, delay precipitation. Uterine fluid in which the large molecules are removed by ultrafiltration has no effect on crystal formation. For further information on the eggshell matrix, see the review by Hincke *et al*. (2010).

Calcium metabolism

The average eggshell contains 2.3 g of calcium and, at a rate of production of 250 eggs per year, the hen turns over 580 g of calcium each year. No other vertebrate animal turns over this much calcium as a percentage of body weight. The calcium used for shell formation comes from the blood. Plasma calcium level rises from 100 μg ml^{-1} before the onset of lay to 200–270 μg ml^{-1} throughout egg production. Most of the calcium in plasma is associated with organic complexes, very-low-density lipoprotein (VLDL) and vitellogenin, with only 20% of the calcium present in an ionized form.

CALCIUM ABSORPTION IN THE INTESTINE An adequate supply of calcium is essential for proper shell formation; and with normal layer rations containing 3.5% calcium, the daily intake is 4.2–4.6 g day^{-1}. Calcium is needed during the night, when shell formation occurs, but the birds are not eating at this time. To compensate for this, birds optimize the absorption of calcium during the early dark period, when feed is still in the gut; calcium absorption is much less efficient when the shell gland is inactive. Feed intake increases in the 2 h before the onset of darkness and hens will readily pick out large particles of oyster shell or limestone pellets and store these in the gizzard, where soluble components will be leached by the HCl present. The passage of calcium through the digestive tract can be regulated to optimize the absorption of calcium. A coarse particle size of calcium improves shell quality by increasing the period when calcium is available from the diet. High levels of saturated fatty acids in the diet lead to the formation of calcium soaps and decrease calcium bioavailability. High levels of dietary phosphorus decrease calcium availability,

with 0.3% available phosphorus being adequate for normal performance and bone integrity.

Calcium is absorbed through the intestinal brush-border membrane via the epithelial calcium channel down a concentration gradient from high levels in the intestinal lumen. Calcium then binds to calbindin, which may act as a transport protein or a buffer to keep free intracellular calcium levels low. This protein is also found in the shell gland (see below and Fig. 4.16). Calcium is then actively pumped from the epithelial cell into the blood via a calcium–ATPase pump and a Ca^{2+}/Na^{+} exchanger. Calcium uptake can also occur via paracellular passive transport through tight junctions between the intestinal epithelial cells. Both of these processes are regulated by vitamin D.

CALCIUM TRANSPORT IN BONE The additional calcium that is required for shell formation during the second half of the dark period (about 20–40%) is mobilized from storage in bone (Fig. 4.15). The calcium required for the formation of a single eggshell is equivalent to about 10% of the total calcium stored in the skeletal system. In the absence of adequate dietary calcium, the bones can be demineralized, causing 'cage layer fatigue' and bone breakage. Calcium storage in bone develops as oestrogen levels increase at sexual maturity and is in a labile form of calcium phosphate, stored in the medullary bone present in the marrow of some long bones. Vitamin D stimulates the mobilization of calcium from bone by osteoclasts, as well as calcium uptake from the intestine by increasing the synthesis of the calcium-binding protein calbindin. Vitamin D_3 is converted into 25-hydroxycholecalciferol in the liver and then further hydroxylated to the active form $1,25(OH)_2D_3$ in the kidney. If blood calcium levels decrease, PTH is released from the parathyroid glands. PTH stimulates osteoclast activity directly and also increases the 1α-hydroxylation of 25-hydroxycholecalciferol in the kidney, which increases the removal of calcium from bone. Calcium deposition in bone occurs by osteoblasts, which have increased activity when the shell is not being deposited, so that the bone store of calcium can be replenished.

Tibial dyschondroplasia (TD) is a lesion that occurs in fast-growing broilers where the growth plate of the tibia head is not mineralized, causing bowing of the tibiotarsus and lameness of the bird. Increasing dietary vitamin D_3 along with increased dietary Ca can reduce the incidence and severity of TD and improve bone health in young broilers.

CALCIUM TRANSPORT IN THE SHELL GLAND During shell formation, the uterine cells secrete a fluid into the shell gland that is supersaturated with calcium

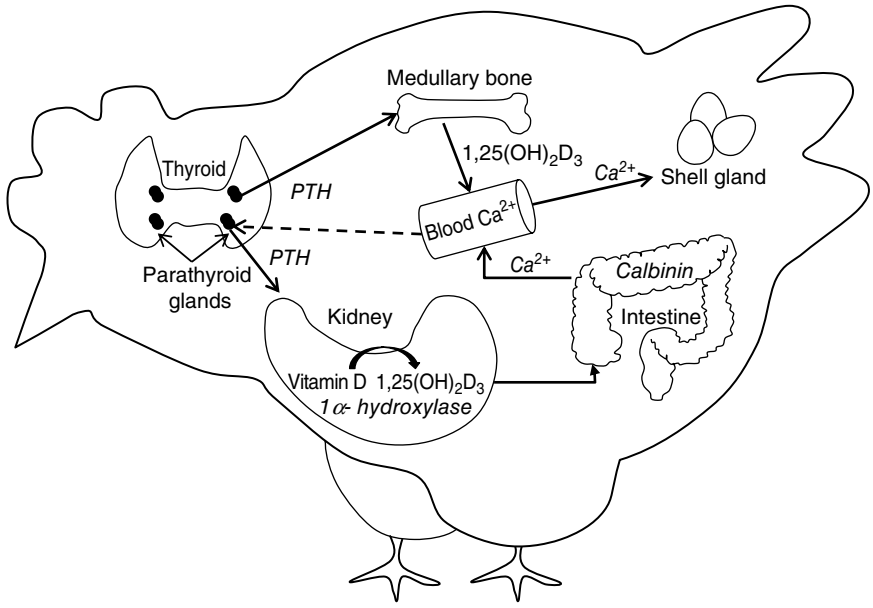

Fig. 4.15. Calcium mobilization in birds.

and bicarbonate. The changes that occur in uterine fluid during calcification are summarized in Table 4.3. Sodium and chloride levels are high in the uterine fluid at the beginning of calcification and then decrease by 18 h as they are reabsorbed by active transport into the uterine cells. The level of potassium increases and the pH decreases during calcification. Calcium absorption from the blood and secretion in the uterine fluid occurs through the uterine glandular cell by active transport via a calcium–ATPase pump and a Ca^{2+}/Na^{+} exchanger (Fig. 4.16). The calcium-binding protein, calbindin, is present in the uterine cells, and this protein is identical to a vitamin D-dependent protein found in the intestine. Calbindin may be involved in Ca^{2+} transport or in protecting the cells from high levels of intracellular calcium. Levels of calbindin increase in uterine cells at sexual maturity in response to oestrogen stimulation of oviduct development and are not dependent on vitamin D. Calcium secretion and levels of calbindin mRNA vary during the ovulatory cycle and increase during shell formation. Levels of calbindin are lower in hens that are not in lay or are producing shell-less eggs or eggs with thin shells. Shell deposition only occurs when the presence of a yolk in the shell gland is synchronized with ovulation. This suggests that hormonal factors linked to ovulation and follicular maturation regulate calbindin synthesis.

Table 4.3. Summary of ionic changes in uterine fluid during shell formation. Values are reported in mmol l^{-1} except for pCO_2, which is in mmHg.

	Start of calcification	End of calcification
Na	144	80
Cl	71	45
K	12	60
Ca	6–10	
HCO_3^-	60–100	
pCO_2	100	
pH	7.6	7.1

Bicarbonate is produced by the action of carbonic anhydrase in the uterine cells (Fig. 4.16) and is used to form calcium carbonate crystals in the uterine fluid. The level of carbonic anhydrase increases with oviduct development and also at the onset of egg production. A vitamin D response element is present in the carbonic anhydrase II gene but the activity of carbonic anhydrase is associated with the development of the oviduct induced by sex steroids. Unlike in the intestine, the transport of calcium in the shell gland is not dependent on vitamin D. However, the active transport of calcium may play only a partial role in eggshell calcification, with some calcium transport occurring by passive paracellular mechanisms, as in the intestine. Amorphous calcium carbonate can also be released from the uterine cells in extracellular vesicles,

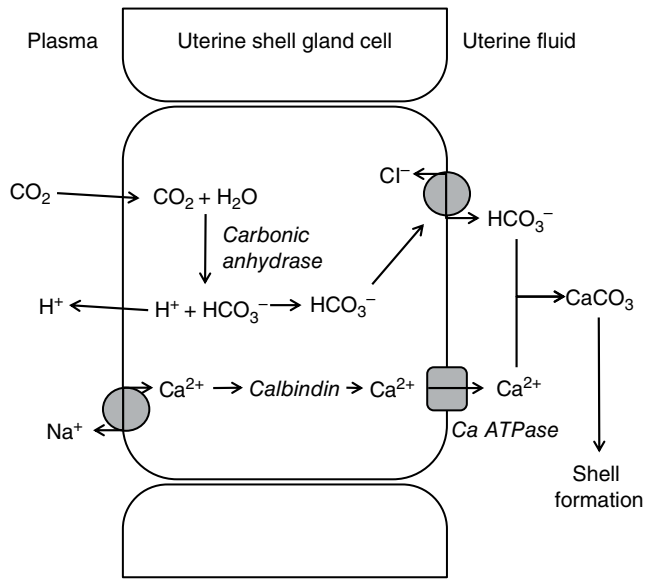

Fig. 4.16. Ionic fluxes through uterine shell gland cells.

which move through the uterine fluid to the mineralization sites on the growing shell. The transport of calcium to the shell gland is thought to be driven by the activity of carbonic anhydrase; due to the high levels of bicarbonate produced, levels of free calcium in the shell gland are low and passive calcium transport to the shell gland is increased. For more information on eggshell mineralization, see the review by Gautron *et al*. (2021).

Chloride levels greater than 0.2% negatively affect shell quality, particularly for older hens. High environmental temperature decreases shell quality because of reduced calcium consumption as well as increased respiration rate, which causes respiratory alkalosis and reduces the CO_2 and $(CO_3)^{2-}$ available for shell formation. Supplying carbonated water, sodium bicarbonate and vitamin C may also improve shell quality, particularly in hot environments. Increasing the concentration of CO_2 in the air also improves shell quality, but this is not practical in commercial egg production. Eggshell quality can also be improved in older hens by including an optimal level and form of manganese, as well as by pre- and probiotics, organic acids and herb extracts.

For interest

Single nucleotide polymorphisms (SNPs) in the carbonic anhydrase II gene are associated with egg shape (Dunn *et al*., 2012).

Applications

The levels of plasma hormones such as PTH, as well as PTH-related peptide, oscillate during the egg cycle and may be involved in regulation of matrix-protein synthesis. However, the hormonal control of matrix-protein synthesis has not been established. Genetic markers for eggshell mineralization and shell quality have been developed. Hens with poor eggshell quality tend to have lower plasma levels of $1,25(OH)_2D_3$ and this could possibly be used in genetic selection programmes for improved shell quality.

4.3 Wool Production and Endocrine Defleecing

Key concepts

- Skin functions as a protective barrier to internal tissues but is also an active endocrine organ.
- Wool (hair) is made up of dead keratinized cells produced by follicles, which undergo a growth cycle.
- *In vitro* and *in vivo* model systems have been developed to study fibre growth in follicles.
- Defleecing can be achieved by imbalances in Cu, Zn or amino acids that arrest fibre growth.
- A large number of growth factors act in an autocrine or paracrine manner within the follicle to regulate cell function.
- Endocrine defleecing can be accomplished in Merino sheep using EGF.

Skin as an endocrine organ

The skin has a major protective function as a physical barrier, separating internal tissues from the external environment and protecting them from damage. Skin is also involved in the regulation of body temperature, in preventing dehydration, in the immune response and in sensing the external environment through nerve endings. The skin consists of three layers: the waterproof outer epidermis composed of stratified squamous epithelium; the dermis, which has connective tissue, blood vessels, nerves and exocrine sweat and sebaceous glands; and the inner hypodermis, which has connective and adipose tissue with an extensive network of blood vessels (Fig. 4.17).

The skin is also an endocrine organ, since it responds to hormones from other glands, regulates hormone action by their activation, inactivation and removal, and generates hormones that act in a local autocrine or paracrine manner within the skin (Fig. 4.18). The epidermis is arranged in epidermal units, which comprise a single melanocyte surrounded by 36 keratinocytes. The melanocytes are responsible for skin pigmentation and protection against the effects of UV light through the production of melanin, which is transferred to the surrounding keratinocytes in each epidermal unit. The melanocytes are stimulated to produce melanin by melanocyte-stimulating hormone (MSH), which is produced in response to ultraviolet-B (UVB) exposure. This system is a homologue of the hypothalamic–pituitary–adrenal (HPA) axis, known as the skin stress response system (SSRS), where UV exposure increases corticotrophin-releasing hormone (CRH) levels in the skin, which signals pro-opiomelanocortin (POMC) release and cleavage to MSH, corticotrophin, β-lipotrophin and β-endorphin in the keratinocytes. Corticosteroid production in the

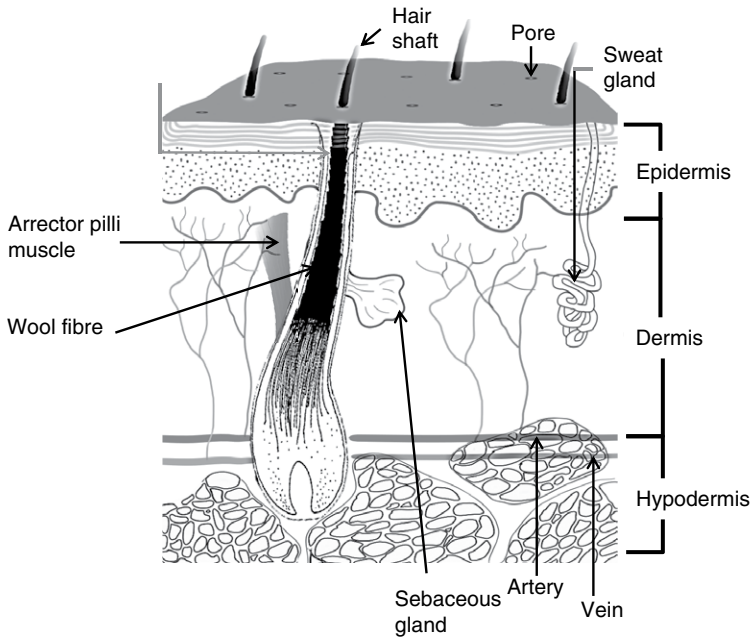

Fig. 4.17. Structure of the skin.

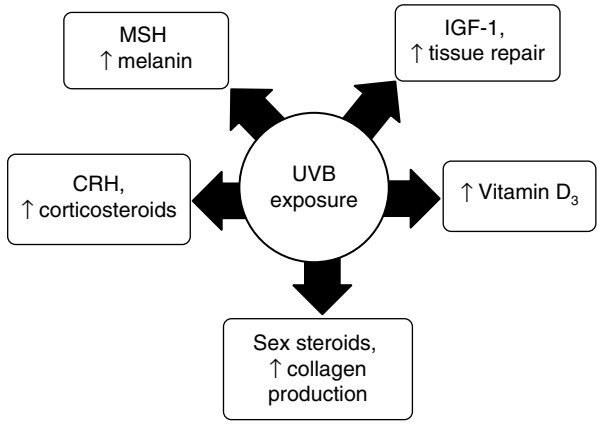

Fig. 4.18. Endocrine activities in the skin.

skin is also increased in response to CRH from UVB exposure. There are two types of CRH receptors in the skin: CRH-R1, which occurs in most cells of all skin layers and is involved in the pathogenesis of many skin diseases; and CRH-R2, which is found in hair follicles and sebaceous glands.

IGF-1 is produced by fibroblasts in the dermis of the skin and is involved in the hair growth cycle and the proliferation and differentiation of hair and wool follicles. It is also involved in the remodelling of tissue and in the response to damage of the epidermis from UVB exposure. UVB-induced cellular damage activates IGF-1 receptors in keratinocytes in the epidermis, which prevents the possible malignant transformation of cells and reduces the incidence of cancer.

The skin is the site of the synthesis of vitamin D. When skin is exposed to UVB, it causes photolysis of

7-dehydrocholesterol (provitamin D_3) to form previtamin D_3, which isomerizes to form vitamin D_3, an inactive prohormone. Vitamin D_3 is bound by a vitamin D-binding protein and transported to the liver, where it is converted to 25-hydroxyvitamin D_3. Following transport to the kidneys, 25-hydroxyvitamin D_3 is then converted to the active form of vitamin D, calcitriol (1α,25-dihydroxyvitamin D_3), in the cells of the proximal convoluted tubules (see Sections 4.1 and 4.2 for more details on vitamin D metabolism).

Sex hormones are converted to their active forms in the skin. In males, skin sebocytes, sweat glands and dermal papilla hair cells express 17β-hydroxysteroid dehydrogenase (HSD) and 5α-reductase enzymes that convert DHEA and androstenedione from the adrenal cortex into testosterone and dihydrotestosterone (DHT). In females, the sebaceous glands, the outer and inner root sheath cells of anagen terminal hair follicles and dermal papilla cells express aromatase that converts testosterone into oestradiol. Human keratinocytes convert oestradiol to oestrone, and fibroblasts and adipocytes produce oestradiol. Epidermal keratinocytes and melanocytes express androgen receptor and oestrogen receptor. Oestradiol and testosterone stimulate collagen production, which maintains the integrity of the extracellular matrix and is essential to the overall elasticity and structure of the dermis. For more information, see Slominski *et al.* (2013).

Wool growth

Wool is considered to be the first textile fibre used by humans for insulation and protection. Wool fibres are hairs, but the term 'wool' is usually used to describe the fine curly hairs that are produced by sheep. It is also used to describe the fleeces produced by other animals; for example, the Angora rabbit and Cashmere goat are highly prized for their wool. The fibre is made up of columns of dead keratinized epidermal cells bonded together by extracellular proteins. The outer layer of flat cuticle cells of the fibre is most heavily keratinized and surrounds an inner layer of cortex cells. The arrangement of the different cell types on the cuticle and within the cortex varies between hairs from different sources.

The value of wool is determined by average fibre diameter, staple strength and clean fleece weight. The major objective of wool producers is to decrease fibre diameter while maintaining the weight of the fleece and this can be achieved by increasing the density of follicles that produce the wool during fetal development. There are two types of follicles in the dermis: primary follicles, which have a sweat gland and erector pilli muscle, are formed first in embryonic development day 70 (E70); followed by secondary follicles at E85 and secondary branched follicles at E105. The total number of secondary and secondary-derived follicles, assessed as the secondary:primary (S:P) follicle ratio, determines the fibre diameter, follicle number and clean fleece weight. Development of secondary follicles in the fetus is reduced by maternal undernutrition and physiological stress, leading to increased levels of plasma cortisol. All follicles have begun to develop and the maturity of primary follicles are complete by birth at E145 to E150, but growth of secondary hair follicles continues for another 4–5 months.

Follicles develop by the proliferation of epidermal keratinocytes to form a placode along with an underlying aggregation of dermal cells, which grow down together into the dermis. The dermal cells then move into the epithelial bud to form the pre-papilla, and the sebaceous gland forms at the side of the follicle. The epithelial bulb cells then envelop the pre-papilla to form the papilla as the follicle lengthens into the dermis and the arrector pili muscle begins to grow.

Fibre growth begins with cells that proliferate in the lower region of the follicle bulb; above that region, cells begin differentiating into cell lineages that will form the fibre shaft and the surrounding sheaths (Fig. 4.19). Up to 80% of the cells differentiate

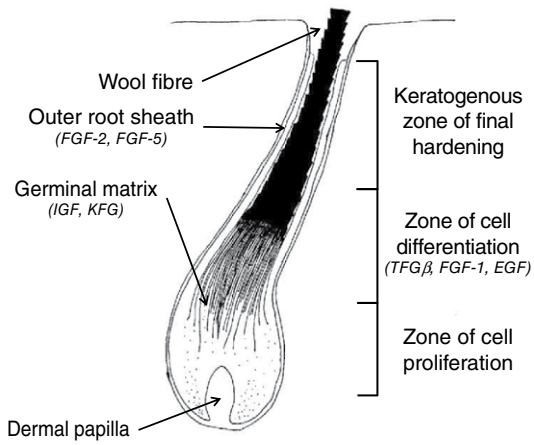

Fig. 4.19. Follicle anatomy and growth factors involved in fibre growth.

to form the inner root sheath, which forms the outer cuticle of the fibre. The remainder form the cortex of the fibre shaft, and these cells produce keratin and other proteins that are the main components of the wool fibre. In the keratogenous zone, cells produced in the bulb grow and are modified to form the wool fibre and root sheath. Oxidation of thiol groups in cysteine residues in proteins occurs here to form disulfide cross-links between proteins. RNA and DNA are degraded and resorbed at the top of this zone. In the zone of final hardening of the wool fibre, oxidation of the thiol groups is completed.

Changes in the cellular activity in the mature wool follicle affect the rate of fibre production and fibre diameter. Follicles undergo a growth cycle, which consists of periods of fibre growth that involves proliferation and differentiation of matrix cells to produce hair shaft (anagen), followed by cessation of proliferation, apoptosis of the lower portion and follicle regression (catagen), quiescence and shortening of the follicle (telogen), expulsion of the old hair from the follicle (exogen) and re-initiation of fibre growth (proanagen). Fibre growth occurs independently in each follicle and this cyclic growth is regulated by growth factors. In Merino sheep, the wool follicles have a long period of anagen, estimated at 2 years, producing fibres of 15 cm in length annually. For more information, see Rogers (2006).

Some ancestral breeds of sheep, such as the Wiltshire Horn, naturally shed their wool seasonally. Shedding occurs during the spring and early summer, when day length is increasing, and there is less melatonin and more prolactin. Prolactin levels are minimal in winter and this results in more constant follicle activity, while growth activity is reduced moving towards the winter solstice with increased exposure to melatonin. Prolactin reacts with the membrane-bound prolactin receptors (PRLRs) on the outer root sheaths and dermal papillae of the follicles to activate the telogen stage and has an inhibitory effect on the anagen phase. Wool growth in other non-shedding domesticated breeds (e.g. Romney) varies seasonally and is correlated with changes in photoperiod, but how the wool growth cycle is impacted differs among breeds.

Defleecing methods

The traditional method of mechanical shearing of sheep is labour intensive, requires considerable skill, is time consuming and is a major annual cost to a wool-growing enterprise. Simple, low-cost and effective methods for defleecing sheep would thus provide considerable benefits.

Copper deficiency results in wool with low intrinsic strength and zinc deficiency results in partial or complete shedding of the wool. Chelating agents such as ethylenediaminetetraacetic acid (EDTA) can be given to reduce the available copper and zinc and cause defleecing, but the doses required are near-lethal. Single oral doses of various thallium salts (10–14 mg kg^{-1} BW) or the anti-cancer drug cyclophosphamide (25–30 mg kg^{-1} BW), which arrests cell division, can cause defleecing, but these compounds are toxic. Hair loss in mice is induced by the selenium-containing amino acids selenocystathione and selenocystine but these compounds have not been tested in sheep, due to their cost and toxicity.

Producing imbalances in available amino acids can also weaken the wool fibres and a lack of essential amino acids will reduce wool growth. This can be done by infusion of an amino acid mixture without lysine or methionine into the abomasum, bypassing any microbial degradation in the rumen. Infusion of methionine into wheat-fed sheep leads to distortion and partial degradation of the wool fibres. Abomasal infusion of a complete amino acid mixture or infusion of methionine into roughage-fed or grazing sheep increases wool growth.

Intravenous infusion (80 mg kg^{-1} BW for 2 days) or oral doses (400–600 mg kg^{-1} BW) of the amino acid mimosine (Fig. 4.20) to sheep decreases wool growth and causes defleecing. Mimosine, which is present in high levels in the tropical legume *Leucaena leucocephala*, arrests cell division in the follicle bulb and wool fibre growth for 10–12 days within 2 days after dosing. The rate of regrowth and fibre diameter are increased after defleecing. Lower doses cause a partial break in the fibres without shedding the fleece. The analogue isomimosine is also effective in defleecing. Mimosine is effective under controlled laboratory conditions but is not completely reliable in practice. A large

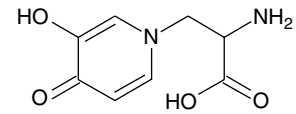

Fig. 4.20. Structure of mimosine, an amino acid that causes defleecing.

dose is needed for defleecing (16 g for a 40 kg sheep), which is difficult to administer, is close to the lethal dose and the effectiveness is influenced by the nutrition of the sheep. For more information on chemical defleecing, see Reis and Panaretto (1979).

Hormones from the thyroid, adrenal, pineal, pituitary and gonads can affect hair and wool loss in mammals. In Wiltshire Horn sheep, the growth cycle can be manipulated by altering photoperiod or prolactin levels. Suppression of prolactin results in the follicles remaining in anagen, while a high level of prolactin induces catagen in the follicles. Administration of ACTH or cortisol reduces the rate of cell division in the follicle bulb to inhibit wool growth but long-term exposure to high levels of hormone is needed. Shedding is induced in Merino sheep with synthetic corticosteroids such as dexamethasone given at 8.5 mg kg^{-1} $BW^{0.75}$ (metabolic body weight) and flumethasone given at 1.3 mg kg^{-1} $BW^{0.75}$ as a constant intravenous infusion over 8 days. Plasma levels of dexamethasone and flumethasone were 40–50 and 8 μg ml^{-1}, respectively, from this treatment. This dose is expensive and the extent of shedding varies among animals and differs in various anatomical areas on the animals.

Endocrine defleecing can be accomplished in Merino sheep using EGF. This process, known as Bioclip®, was developed by CSIRO Animal Production and The Woolmark Company in Australia and was marketed in Australia from 1998. In the first stage, known as donning, sheep are prepared by removing undesirable fleece, such as head and shank wool or stained wool, to improve the quality of the final wool that is harvested. The sheep are then fitted with a fleece-retention net and injected with EGF in the inguinal (inside back leg) region. Fleece detachment occurs at day 7 after EGF treatment. The net is removed along with the wool from day 28, when there is sufficient wool regrowth to protect the sheep from climatic extremes.

Endocrine defleecing has the advantages of improving wool quality by eliminating second cuts on the wool, thus reducing the variability in fibre length, the amount of short fibres and mechanical damage, and improving carding yield. The ends of the wool fibres are more rounded and thus the wool is softer and more comfortable than wool harvested by mechanical shearing. The defleecing process does not affect non-wool fibres, such as kemp and hair fibres, which remain on the sheep and do not contaminate the final fleece. This improves the processing and dyeing of the wool yarn. Avoiding shearing cuts to the skin also decreases the contamination of the wool with skin pieces and increases the value of the sheep leather. There is also less stress and injury hazards for both the sheep and the handlers, who do not have to be skilled shearers.

Commercial use of EGF for endocrine defleecing (Bioclip®) uses urogastrone epidermal growth factor (URO-EGF), which is a structural analogue of human EGF produced by Anagen Pty Ltd and available from Heiniger Australia Pty Ltd in Australia. However, the technology was not widely accepted, likely due to high cost, the need for a net to retain the fleece and it cannot be used on pregnant animals. Anagen Pty Ltd was dissolved in 2017.

Model systems used to study function of follicles

A simplified *in vitro* model system to study follicular function is the microdissection of follicles from skin samples, followed by growth in culture (for example, see Bond *et al*., 1998). *In vivo* model systems in which the follicle activities are exaggerated, such as synchronized cycles of shedding sheep, can also be used. Whole animals can be treated with growth factors and depilation (removal of wool) force measurements are then used to determine if the strength of the wool fibres has been affected. Skin samples can be removed from the animal after treatment and various cell parameters can be measured. Skin xenografts can also be made to severe combined immunodeficiency (SCID) mice, which will not mount an immune response against the graft. Growth factor expression can be knocked out or growth factors can be over-expressed in transgenic animals. Naturally occurring mutations that affect fibre growth, such as angora, waved and rough, can also be studied to determine the role of growth factors in these phenotypes.

Growth factor effects on hair and wool follicles

A large number of growth factors have been implicated in the regulation of wool follicle function (Table 4.4 and Fig. 4.19). These factors act locally within the follicle to regulate cell function in an autocrine or paracrine manner. Growth factors

Table 4.4. Growth factors affecting follicular function (after Nixon and Moore, 1998).

Growth factor	Receptor(s)	Function
Insulin-like growth factor-1 (IGF-1)	IGFR-1, insulin receptor	Required for follicle growth in culture; expression varies with follicle cycle
Insulin-like growth factor-2 (IGF-2)	IGFR-1 and 2	IGF-2 mRNA and receptor present in follicles
Acidic fibroblast growth factor (FGF-1)	FGFR-1	Associated with differentiating keratinocytes
Basic fibroblast growth factor (FGF-2)	FGFR-2	Associated with basement membrane of follicle; inhibits hair growth in mice
Keratinocyte growth factor (FGF-7 or KGF)	FGFR-2	Dermal papilla to germinal matrix communication
Fibroblast growth factor-5 (FGF-5)	FGFR-1	Cycle-dependent expression in peripheral follicle cells
Transforming growth factor-β (TGFβ)	TGFβR-I and II	Follicle regression and inhibition of cell proliferation
Epidermal growth factor (EGF)	EGFR	Inhibits cell proliferation in wool follicle and causes catagen in follicle and fibre weakness
Transforming growth factor-α (TGFα)	EGFR	Mutants have enlarged follicles and wavy fur
Hepatocyte growth factor (HGF)		Keratinocyte growth and motility
Parathyroid hormone-related protein (PTHrP)	PTHR	Antagonists stimulate hair growth in mouse
Platelet-derived growth factor (PDGF-A and B)	PDGF-Rα and β	Mitogenic in connective tissue sheath of hair follicles
Vascular endothelial growth factor (VEGF)		Autocrine action in human dermal papilla cells

bind to cells within the wool follicle bulb to affect cell proliferation and keratinocyte differentiation, which affect the rate of growth and characteristics of the wool fibre. Growth factors also bind to the extracellular matrix (ECM), which consists of fibrous proteins, glycoproteins and proteoglycans. Growth factors influence ECM synthesis and degradation or are activated by ECM components and affect the wool follicles embedded in the ECM.

Growth factors affect the morphogenesis of wool follicles in the fetus and also the proliferation and differentiation of cells in the mature follicles. The development of follicles in the fetus determines the density, size and form of follicles, which affects the extent of wool production and wool fibre characteristics in the adult.

Insulin-like growth factors

The insulin-like growth factors are single-chain polypeptides of about 70 amino acids. They act as mitogens, morphogens and differentiation and cell-cycle progression factors. ST stimulates IGF-1 production by liver and peripheral tissues, as discussed in Section 3.4. IGF-2 is present in higher amounts than IGF-1 in sheep plasma and skin but its role is less well understood.

Insulin-like growth factors stimulate keratinocyte proliferation and prolong the growth phase of hair follicles. IGF-binding proteins have been found in the dermal papilla, suggesting that they may modulate the effects of IGF on fibre growth. Mice with null mutations for IGF-1 and IGF-2 have hypoplasia of the epidermis and reduced follicle density. Conversely, mice over-expressing IGF-2 had skin hypertrophy, while transgenic sheep expressing IGF-1 had improved wool production. However, whole-body or skin-patch infusions of IGF-1 in sheep had no effect on wool growth.

Fibroblast growth factors

The key members of the FGF family involved in the maintenance and control of fibre growth are acidic FGF (FGF-1), basic FGF (FGF-2), keratinocyte growth factor (KGF or FGF-7) and FGF-5 (see Fig. 4.19). Basic FGF is found in the outer root sheath of the wool follicle and is associated with the ECM between the outer root sheath and the dermis. It may provide a mitogenic stimulus for the follicle bulb cells. Acidic FGF is concentrated in the upper bulb cells and is associated more with cell differentiation than proliferation. It appears to regulate fibre differentiation by affecting the expression of particular keratin genes and the pattern of differentiation of cells in the follicle. KGF is synthesized within the dermal papillae, while its receptor is present in epithelial follicle cells in the bulb. Treatment with KGF stimulates hair growth in mice, while KGF knockout mice have matted and greasy coats, similar to the naturally occurring rough mutation. KGF is thus important for normal fibre development. FGF-5 is

localized to the outer root sheath and is increased during anagen and decreased after catagen. Gene knockout resulted in an exceptionally long coat, analogous to the angora mutation. FGF-5 thus appears to be involved in follicle regression.

Transforming growth factor-β

The TGFβ family includes three isoforms of TGFβ along with bone morphogenic proteins (BMPs) and the inhibins and activins. They are synthesized as a large precursor that is cleaved to form a 112 amino acid growth factor and a binding protein. TGFβ receptors are serine/threonine kinases (see Section 1.3). Binding of the growth factor to the Type II receptor results in phosphorylation and activation of the associated Type I receptor. The receptors are located in the follicle matrix and outer root sheath. TGFβs regulate cell growth and differentiation and are some of the earliest signals found during development of skin and hair follicles. They generally inhibit growth by suppressing mitosis of epithelial cells, inducing differentiation, or inducing apoptosis. The roles of the individual TGFβs are not clear, but they are involved throughout follicle morphogenesis. Transgenic over-expression of BMPs in the outer root sheath and TGFβ1 in the epidermis results in decreased epithelial cell proliferation and follicle formation. TGFβs also regulate cell proliferation in follicles during the hair growth cycle, with highest levels at catagen.

EGF family of growth factors

Skin and hair follicles synthesize a number of the EGF family of peptide growth factors (Table 4.5), including EGF, TGFα, amphiregulin and heparin-binding EGF, which all bind the EGF receptor. Members of the EGF growth factor family have a 36–40 amino acid conserved motif and three disulfide bridges, which form three peptide loops. EGF stimulates cell proliferation, while TGFα may be the fetal ligand for the EGF receptor and results in a transformed phenotype by regulating cell migration and differentiation. Amphiregulin contains a putative nuclear translocation signal and stimulates the growth of normal keratinocytes and fibroblasts, while inhibiting growth of several breast cancers. Heparin-binding EGF has an N-terminal extension and can bind the extracellular matrix. Cripto is another member of the EGF family of growth factors. It induces branching morphogenesis in mammary epithelial cells and inhibits the expression of various milk proteins.

EGF stimulates the proliferation of cell types of ectodermal or endodermal origin, but in the hair follicle it induces catagen. EGF consists of 53 amino acids but it is also active as a 150 amino acid prohormone when it is anchored to the cell membrane with the EGF sequence located in the extracellular space. The EGF peptide is identical to urogastrone, which is a peptide isolated from the urine of pregnant women that blocks the secretion of gastric juices. A major site of EGF production is the salivary gland and production is stimulated by androgens. EGF is released in the saliva and is thought to play a role in wound healing in animals that lick their wounds. The studies using EGF to inhibit wool growth used EGF prepared from submaxillary glands of adult male mice. The activity of EGF is measured with a bioassay that measures its effectiveness in causing precocious incisor eruption and eyelid opening in newborn mice. EGF was first named based on the thickening and keratinization

Table 4.5. EGF superfamily of growth factors.

EGF type	Receptor type	Function
EGF (urogastrone)	ErbB1 (EGFR)	Promotes mitosis in mesodermal and ectodermal tissue; inhibits secretion of gastric acid
TGFα	ErbB1	Transforms normal cells; proposed fetal ligand for EGFR
Amphiregulin	ErbB1	Stimulates growth of normal fibroblasts and keratinocytes; inhibits growth of carcinoma
Neu differentiation factors (NDF), neuregulins and hereregulins	ErbB3 and ErbB4	
Betacellulin	ErbB1 and ErbB4	
Epiregulin	ErbB1 and ErbB4	
Heparin-binding EGF	ErbB1 and ErbB4	
Cripto		Mammary gland development

of the epidermis on the back and eyelid caused by injection of salivary gland extracts.

EGF RECEPTOR The EGF receptor (EGFR) is a 180 kDa polypeptide that is part of a larger family of receptors known as ErbB (a term derived from erythroblastic oncogene B) which are the receptors for the *v-erb-B* oncogene (see Table 4.5). The EGFR was identified in 1980 as the first member of the tyrosine kinase receptor type (see Section 1.3). Shortly afterwards, the receptors for insulin, IGF-1 and PDGF were also shown to be tyrosine kinase receptors. The free receptor is a single polypeptide chain. Binding of EGF to the receptor causes the receptor to form a dimer, which is in many cases a heterodimer of different receptor subtypes. Whether a homodimer or heterodimer is formed and which receptors are involved dramatically affects the signalling properties of the receptor.

When the dimer is formed, the catalytic site of one chain phosphorylates several tyrosines in the adjacent chain. These phosphotyrosines serve as recognition sites for binding of signalling proteins that have an SH2 domain. An example of these signalling proteins is phospholipase C (PLCγ1), which is activated by binding to the receptor, thereby increasing calcium mobilization and activating protein kinase C. PLCγ1 also has an SH3 domain, which allows it to bind to the cytoskeleton and provide access to the phosphatidylinositol substrate in the cell membrane. EGFR signalling is involved in regulating cell development and motility, protein secretion, wound healing and tumorigenesis.

EFFECTS OF EGF ON FOLLICLES EGF reduces hair growth in mice by reducing the number of proliferating cells in the follicle bulb and reducing the rate of follicle development. Mice treated with EGF produce hair that is shorter, finer and wavy. Inactivation of the TGFα gene in knockout mice also produces a wavy coat, while over-expression of this gene results in a thickened epidermis and stunted hair growth.

EGF treatment of sheep produces different effects in the follicle than in the surrounding epidermal cells. EGF treatment causes weakening in the unhardened wool fibres and a partial disruption of fibre growth in the inner root sheath cells in the keratogenous zone of the follicle bulb. A gradual inhibition of mitotic activity then occurs in the bulb cells over a period of 2–3 days, with a catagenic regression of the follicle. This is followed by asynchronous regeneration of the follicles 4–8 days after infusion of EGF. However, EGF has the opposite effect on cells in the epidermis and sebaceous gland, where cell proliferation is increased by 2 days after infusion of EGF. The mechanism behind the contrasting effects of EGF in the follicles and epidermis is not known. It may be the result of a massive disruption in the normal homeostatic mechanisms that regulate the activities of the different cell types in the skin.

Subcutaneous injections or intravenous infusions of 3–5 mg of EGF caused a transient regression of the wool follicles from the actively growing stage and resulted in shedding of the entire fleece. Treatment with 1–3 mg of EGF resulted in a zone of weakness in the wool fibres 3–4 weeks later, but the fleece was not shed. The weakness of the wool fibres is assessed by measuring the depilation force, which is the force required to pluck wool staples from the mid-back region of the sheep.

Following treatment with EGF, there were changes in the regrowth wool. The content of high-sulfur proteins increased from 19% to 30%, while the content of high-tyrosine proteins decreased from 12% to 5%. There were also alterations in the proportions of other components. The maximum change in composition was observed around 4 weeks, with the composition returning to normal by 10 weeks. The effects were dose dependent and were similar to those seen when chemical defleecing agents were used. This suggests that the changes in wool composition are characteristic of wool growing from newly regenerated or regenerating follicles.

OTHER EFFECTS OF EGF Other effects of EGF are the requirement of sufficient time for regrowth of the wool, food rejection and anorexia, decreased gastrin secretion and gut motility, and an increase in abomasal pH. There can be erythema around the eyes and muzzle and some rupturing of dermal blood vessels. In pregnant ewes, there can be a delayed onset of oestrus, abortions, increased ST and placental lactogen, and decreased thyroxine but no effect on pregnancy rate or lambing. In rams, there can be decreased sperm motility for about 12 weeks and increased plasma cortisol. Because of the negative effects of EGF on reproduction, it is recommended that producers wait 5 weeks after using EGF before breeding.

Summary of growth factors affecting fibre growth

FGF-5 and TGFβ have been identified as markers of catagen and they inhibit follicle growth. EGF and FGF can influence fibre characteristics. Fibre curvature may be controlled by factors that result in an asymmetric distribution of keratinocytes in the follicle bulb. Infusion of EGF stimulates epidermal thickening and also induces catagen in wool follicles prior to fleece shedding. For practical applications, it is easiest to treat all sheep about 12 weeks prior to breeding. EGF treatment does produce a variety of behavioural and physiological side effects. For further information, see Popescu and Höcker (2007) and Galbraith (2010).

Questions for Study and Discussion

Section 4.1 Mammary gland development and milk production

1. Describe the structure of the mammary gland. Outline the structural changes that occur during sexual development, pregnancy and the drying off period. What *in vitro* and *in vivo* model systems are used to study mammary gland function?
2. Describe the endocrine factors that regulate mammogenesis. How can nutrition of the prepubertal heifer affect milk yield at maturity?
3. Describe the endocrine factors that regulate the initiation of lactogenesis. How can hormones be used to initiate lactation?
4. Describe the factors that regulate galactopoiesis. How does milk removal increase milk yield?
5. Describe the role of bST in increasing milk production. What are the factors of concern for use of bST to increase milk yield and how can they be addressed?
6. Describe factors that can affect the content of milk protein and milk fat. To what degree can the components of milk be altered?
7. What is the lactocrine hypothesis? What bioactive compounds are present in milk and colostrum?
8. Describe factors affecting ketosis and milk fever. Why are these metabolic diseases more prevalent in multiparous cows?
9. What methods are used to control the metabolic diseases of lactation? What areas need further study in order to develop new methods to control them?

Section 4.2 Egg production

1. Describe factors regulating sexual differentiation and gonad development in birds. Compare and contrast the hormonal and genetic regulation of sexual development in birds and mammals.
2. Describe the hormonal regulation of follicular development in poultry. How does this differ from follicular development in cattle?
3. Outline a potential method to increase egg production and a potential method to increase eggshell strength.
4. Describe the process of moulting to regenerate a flock of laying hens.
5. Describe the structure of the eggshell and the role of the shell matrix. Describe how this can be used as a model system to study calcification in other tissues.
6. Discuss the regulation of calcium metabolism in shell formation. How can you increase the amount of calcium available for shell formation?

Section 4.3 Wool production and endocrine defleecing

1. Describe the structure of skin and the development of wool follicles. How does skin act as an endocrine organ?
2. Describe various nutritional and endocrine methods for defleecing sheep. What are the advantages and disadvantages of each method?
3. Describe the model systems that can be used to study the function of wool follicles.
4. Outline the roles of growth factors, particularly IGFs, FGFs and TGFβ, in growth and differentiation of wool follicles.
5. Describe the roles of the EGF family of growth factors and their receptors in the function of wool follicles.

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5 Endocrine Manipulation of Reproduction

This chapter reviews hormonal manipulation of reproduction in mammals and farmed fish, while aspects of reproduction in poultry are covered in Chapter 4. Sexual differentiation and maturation in mammals are first described, including sexual differentiation of different tissues, the genes that are involved in these processes and the regulation of meiosis in germ cells. The regulation of the oestrous cycle and methods for the manipulation of the oestrous cycle are then discussed. This is followed by a discussion of pregnancy and parturition, and methods for maintaining pregnancy or inducing parturition. Problems that occur during the postpartum interval, inducing puberty, advancing cycling in seasonal breeders and the immunological control of reproduction are then discussed. Endocrine manipulations in aquaculture are then covered, including the control of reproduction by sex reversal and induction of spawning, the endocrine effects on growth and nutrient utilization, and the effects of stress on reproduction, growth and immune function.

5.1 Manipulation of Reproduction in Mammals

This section focuses on the potential for the endocrine manipulation of reproduction in mammals, with a particular emphasis on females, since with the advent of artificial insemination this is usually the limiting factor in animal production systems. Unifying concepts are first presented, followed by discussion of species-specific information, as appropriate. This information is then used as background to understand currently available methods for manipulating the reproductive system and for devising potentially useful methods for future application.

Sexual differentiation and maturation

Key concepts

- The genetic sex directs the development of either ovaries or testes, which then determines the phenotypic sex as the animal matures.
- The undifferentiated mammalian brain is inherently female, but oestrogens programme male behaviour and activity of the hypothalamus. The fetal female brain is protected by the feto-neonatal oestrogen-binding protein (FEBP).
- The pattern of sexual differentiation and development is somewhat species specific.
- The key male sex-determining genes in mammals are *SRY* (sex-determining gene on Y), *SOX9* (SRY box 9) and *AMH* (anti-Müllerian hormone).
- In the absence of *SRY*, ovary development is driven by β-cantenin, *FOXL2* (forkhead transcription factor 2) and *WNT4*.
- Spermatozoa are produced continuously from a population of spermatogonia.
- All the available oocytes are present at birth as a pool of primordial follicles that are arrested in the late prophase or diplotene stage of meiosis. Meiosis is triggered at puberty by increased levels of the gonadotrophins.
- Steroidogenesis is regulated by the pituitary gonadotrophins and somatotrophin.

Sex determination is controlled by the genetic sex of the animal, which is fixed at the time of conception. In the process of sex differentiation, the genetic sex directs the development of either ovaries or testes, which then determines the phenotypic sex by the production of hormones as the animal matures. Usually, a male genotype will produce a male phenotype and a female genotype will produce a female phenotype. However, in some fish and reptiles

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the phenotypic sex can differ from the genotypic sex, due to environmental factors such as temperature. The administration of sex steroids at a critical point during development can also permanently alter the phenotypic sex in some species.

There is usually one pair of sex chromosomes, either XX/XY or ZW/ZZ, for female/male determination. In the XX/XY system found in mammals, the female is the homogametic sex, while in the ZW/ZZ system, the male is the homogametic sex and the female is the heterogametic sex. The ZZ/ZW system (see Section 4.2) is found in birds, reptiles and some fish and amphibians. Other systems of polygenic sex determination involving multiple chromosomes with sex-determining genes are also known in some lower vertebrate species. Polygenic sex determination produces male:female ratios that are different from the 1:1 ratio seen with pure sex chromosome systems.

Differentiation of the gonads and ducts

Sexual differentiation relates to the development of the phenotypic sex from the genetic sex. The primordial gonad consists of supporting cells, steroidogenic cell precursors and primordial germ cells. In the developing testis, the supporting cells form Sertoli cells, which enclose germ cells and form testis cords, while the steroidogenic cells differentiate into Leydig cells. In the developing ovary, the supporting cells form pre-granulosa cells, while the steroidogenic cells become thecal cells and the germ cells that differentiate into oogonia. The supporting cell lineage is the first to differentiate and this channels other cell lineages down either the ovarian or the testicular pathway. This includes the migration of primordial germ cells, the development of gonadal ridges and the differentiation of the gonads into ovaries or testes. The sex-determining gene on Y (*SRY*) directs the formation of testes and the male phenotype; otherwise in the absence of *SRY* the undifferentiated gonads in the early embryo develop into ovaries and lead to a female phenotype. In mammals, organization of spermatic cords during testicular development begins earlier in gestation than organization of the primordial follicles during ovarian development.

In the undifferentiated stage of gonadal development, both Wolffian and Müllerian ducts are present and these later develop into the male or female accessory sex organs (Fig. 5.1). The Müllerian ducts

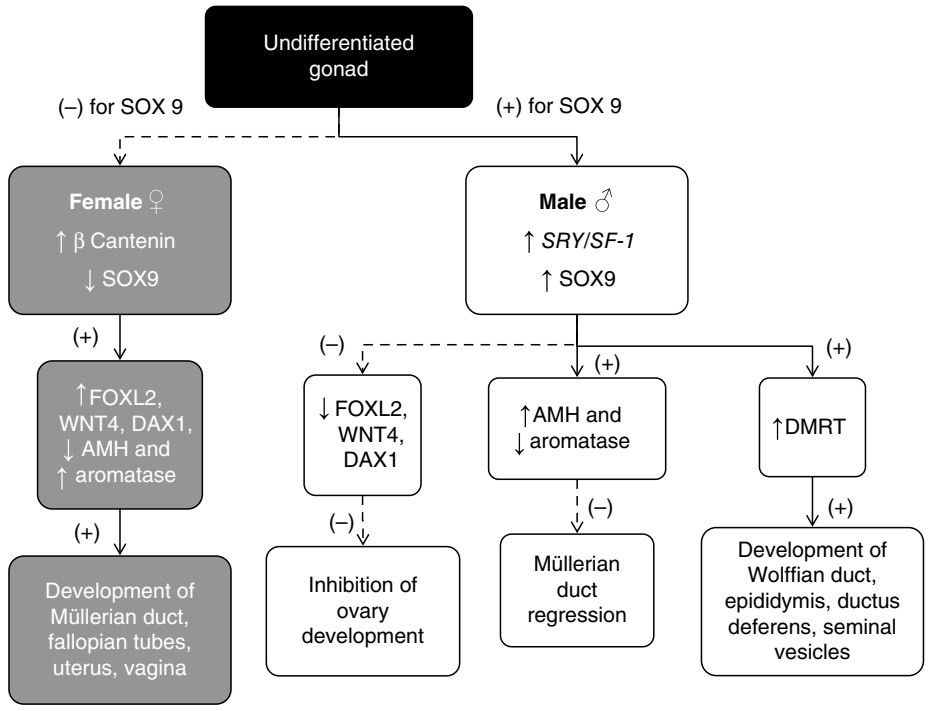


Fig. 5.1. Genetic control of gonadal differentiation in mammals.

differentiate to form the Fallopian tubes, uterus and vagina, while the Wolffian ducts form the epididymis, ductus deferens and seminal vesicles. The Sertoli cells of the fetal testes produce AMH (anti-Müllerian hormone), which is a glycoprotein related to the TGF-β family of peptides. AMH acts locally to induce regression of the Müllerian ducts and to inhibit the expression of aromatase, but it is not required for testis determination (see also Section 4.2 for information on sexual development in birds). The development of male external genitalia is dependent on androgens produced by the testis. In the absence of androgens, the female phenotype develops. Testosterone is converted to the ultimate androgen, DHT, by the 5α-reductase enzyme to stimulate the differentiation of male genitalia. In addition to the androgenic effects of testosterone and DHT, these steroids also produce anabolic effects on various tissues (see Section 3.2).

Differentiation of the brain

The undifferentiated mammalian brain is inherently female but androgens act on the brain to programme male behaviour and the activity of the hypothalamus in controlling pituitary function. Sexual differentiation is a permanent change due to gonadal steroids, which alters the sensitivity of a behaviour to activation by sex steroids later in life. Sexual differentiation of behaviour includes both masculinization, which is the development of male behaviours such as mounting and copulation, and defeminization, which is the loss of sexual receptive behaviour, lordosis and the surge of LH in response to oestradiol. The release of pituitary trophic hormones, such as somatotrophin (ST) and the gonadotrophins, is pulsatile in females and more uniform or tonic in males (see Section 3.4).

The neonatal testis produces androgens, which act at a critical time in development to cause epigenetic changes and programme or 'imprint' a male pattern of function in the hypothalamus, a process known as 'organization-activational programming'. This critical organizational period, which is species specific, may be related to the stage of neuronal development in the hypothalamus and can be affected by thyroid hormones. Generally speaking, in animals with longer gestation periods, which are more developed at birth, the critical period occurs prenatally, while for those with shorter gestation periods, which are less mature at birth, the critical period extends into the early postnatal period. The sexual dimorphic development of the brain results in permanent differences in 'hard wiring' of the brain. This includes differences in neuronal growth, cell death, synthesis of neurotransmitters and the synaptic connections of nerve cells, and in the size of particular regions such as the sexually dimorphic nucleus of the preoptic area (POA). The POA is involved in regulating the release of gonadotrophins due to feedback from oestrogen and progesterone. For characteristic male-dependent behaviors, a second spike in steroid hormones at adulthood (the activational period) is required.

The effect of androgens on the differentiation of the hypothalamus actually occurs via oestrogens, which are synthesized from testosterone by the aromatase enzyme in the brain and bind to oestrogen receptors (ERα and ERβ) (see Section 1.2). The administration of dihydrotestosterone, which cannot be aromatized, does not masculinize the brain but exogenous oestrogens will have this effect. The fetal female brain is protected from the minor levels of circulating oestrogens by the feto-neonatal oestrogen-binding protein (FEBP), which is present at high levels in the circulation. FEBP has a high specific binding affinity for oestrogen and prevents it from reaching the brain. Synthetic oestrogens that have a low affinity for FEBP will also masculinize the brain. In birds, oestrogen results in a female pattern of brain differentiation, while androgens are important in the control of song in male birds. While oestrogens are required for ovary development in non-mammalian vertebrates, they are not required for the initial differentiation of the reproductive tract in mammals.

The development of several other tissues is also sexually dimorphic. For example, there are marked sex differences in steroid metabolism in the liver and this can affect the metabolism and clearance of drugs, hormones and xenobiotics. In rats, the pattern of steroid metabolism in the liver is regulated by the pattern of growth hormone (ST) secretion from the pituitary, and removal of the pituitary results in a male pattern of steroid metabolism. The pattern of ST secretion is due to imprinting of the hypothalamus by exposure to androgens during neonatal life. A discrete pulsatile pattern of ST release occurs at about every 3.5 h in male rats and can be mimicked by intermittent injections. In female rats, ST secretion is more frequent, resulting in a more constant but lower release of ST, which can be mimicked by a constant infusion technique. ST regulates the expression of various

steroid-metabolizing cytochrome P450 (CYP) genes in the liver, by a mechanism that involves coordinate action by transcription factors STAT5b and HNF4α. For more information, see the review by Waxman and Holloway (2009).

Sex differentiation in cattle, sheep and pigs

Sexual differentiation occurs prenatally in sheep and cattle but is completed postnatally in pigs. The pattern of secretion of testicular steroids differs among cattle, sheep and pigs. Boars have three periods of elevated steroids during development, while rams and bulls have only two.

Differentiation of the gonads in cattle occurs from day 45 to day 70 of gestation. Female fetuses that are treated with exogenous testosterone show masculinization of the gonads. Sexual differentiation of the brain in cattle occurs after day 60 of gestation, as treatment with exogenous testosterone before this sensitive period does not affect brain function later in development. Steroid synthesis in female ovaries is low from day 70 to day 85, while levels of androstenedione are high in male fetuses until day 100, thus providing steroid precursors for male programming of the hypothalamus.

Testosterone production by the fetal testis in sheep increases from day 35 of gestation to a peak at day 70. Masculinization of the gonads occurs from day 35 to day 45, while male differentiation of the brain occurs from day 50 to day 80. The reduced steroid production by the ovary from day 45 to day 80 protects the female brain from masculinization. Male lambs need additional exposure to sex steroids during the critical period from 4 to 8 weeks after birth in order for male sexual differentiation to be completed.

Boars have three periods of enhanced testicular development. Levels of testosterone are increased from day 35 to day 40 of gestation, and differentiation of the gonads is complete by day 45. The second period occurs shortly after birth, when the number of Leydig cells increases. During this early postnatal period, the testis is actively producing steroids, due to a lack of negative feedback control of steroids on LH secretion in males at this time. The third period of testicular development occurs at puberty when spermatogenesis occurs. Boars castrated within the first 2 months after birth show female behaviours after treatment with oestrogen as adults, including receptivity to mature boars. However, males castrated after 6 months of age show little female behaviour after oestrogen treatment. This suggests that male programming of the brain in boars occurs during the prepubertal period at 3–5 months of age.

Over 90% of female twins from mixed-sex pregnancies in cattle are freemartins and the syndrome is also known in goats, sheep and pigs. A freemartin is a sterile XX/XY intersex produced from a female twin that has a placenta that is fused with a male twin. The blood systems of the embryos are joined through vascular anastomoses in the fused placenta of the twins. This allows the transfer of AMH, androgens and possibly blood cells from the male to the female fetus, interfering with development of the ovaries and paramesonephric ducts. The gonads of the freemartin range from modified ovaries to testis-like structures that can be retained in the abdomen. The hypothalamus is also masculinized by exposure to androgens and the animal can display male patterns of behaviour.

The risk of freemartinism is greatly increased in sheep with litters of four or more lambs. Placental fusion occurs rarely in mice, as a band of connective tissue separates the two placentas and prevents the formation of a freemartin. This may be a protective mechanism that has evolved in litter-bearing species and may explain the very low incidence of freemartins in pigs. For more information on sex differentiation in cattle, sheep and pigs, see the review by Ford and D'Occhio (1989).

Sex-determining genes

The key sex-determining genes *SRY* and *SOX9* (SRY box 9) have been identified in mammals. These genes code for transcription factors that are involved in testis formation and in the development of the male phenotype. In the absence of the *SRY* gene, ovaries develop. *SRY* is located on the short arm of the Y chromosome and codes for the testis-determining factor protein SRY, which has an 80 amino acid high-solubility group-binding (HMG) domain that can bind to and bend DNA up to 90°. This region also binds calmodulin (see Section 1.3) and contains nuclear localization signals. Mutations in the HMG domain are found in some XY sex-reversed individuals, and CRISPR/Cas-mediated knockout of the HMG domain, followed by a frameshift mutation of the downstream *SRY* sequence, resulted in the development of genetically male (XY) pigs with complete external and internal female genitalia (Kurtz *et al*., 2021). In mice, SRY

also has a large glutamine-rich repeat domain that is involved in transcriptional activation. *SRY* is expressed for a short time during gonad development (depending on the species) and triggers the differentiation of Sertoli cells, the migration of primordial germ cells from the mesonephros to the genital ridges and the proliferation of cells within the genital ridges. SRY acts synergistically with the nuclear receptor steroidogenic factor-1 (SF-1) to trigger each of these events by activating a secondary gene, *SOX9* (see Fig. 5.1). Once it is activated, *SOX9* expression is then maintained by positive regulatory loops to increase the production of Sertoli cells and form a testis. In the absence of SRY, *SOX9* is not induced and the development of the follicle granulosa cells and ovary occurs.

SOX9 is up-regulated in the genital ridges of male embryos and down-regulated in females. The SOX9 protein also has an HMG domain and two transcription activation domains and binds to the regulatory region of the *AMH* gene to increase its expression and promote the regression of the Müllerian ducts. SF-1 plays a role in regulating *SOX9* expression and in the activation of *AMH* gene expression by SOX9. In mammals, the levels of SF-1 are maintained in males but not in females after gonadal differentiation has begun. SOX9 and SF-1 act to repress the expression of genes leading to ovary development, such as *WNT4* (wingless-related integration site, family member 4), *DAX1* (dosage-sensitive sex-reversal (DSS) adrenal hypoplasia congenita (AHC) critical region on the X-chromosome, gene 1) and *FOXL2* (forkhead transcription factor 2), and stimulate the expression of genes involved in defining the Sertoli cell phenotype during testis differentiation. The *DMRT1* (DM domain-related transcription factor 1) gene is related to sex-determining genes in other species and is expressed in the developing testis in XY males. It is also present on the chicken Z chromosome and increased expression of *DMRT1* drives testis development in birds (see Section 4.2).

The formation of the ovary is driven by β-cantenin, which interferes with the maintenance of the expression of *SOX9*. *FOXL2* is involved in the postnatal repression of *SOX9* to maintain follicle (granulosa) cell differentiation. The *WNT* genes code for growth and differentiation factors that play a role in the development of the ovaries and oocytes. WNT4 may act as a suppressor of Leydig cell function in females and is necessary for the formation of Müllerian ducts. WNT4-deficient females show a partial sex reversal. WNT-7α is involved in the further development of the Müllerian ducts to form the oviduct and uterus. *DAX1* is expressed more in females than in males. Both *WNT4* and *DAX1* were originally described as anti-testis factors but have now been shown to be necessary for both ovary and testis development, depending on their level of expression. For more information sex determination, see the review by Estermann *et al*. (2020).

Regulation of meiosis in germ cells

Male gametes, or spermatozoa, are produced from a continuously replenishing population of spermatogonia. Meiosis begins in male germ cells at puberty and continues throughout the reproductive life of the animal. This ensures an abundant supply of male gametes for fertilization. In contrast, the process of folliculogenesis in females produces only a restricted number of oocytes, with meiosis occurring during specific stages of development. The fetal ovary contains all the available oocytes at birth, as a pool of primordial follicles that are arrested in the late prophase or diplotene stage of meiosis. These follicles begin to grow again as the female nears puberty and ovulation begins. During development of the gonads, the fate of the germ cells depends on the surrounding cells in the tissue. Oocytes in the cortex of the developing ovary are the first to reach the diplotene stage and be enclosed in follicles. In the testis, the pre-spermatogonia are enclosed in testicular cords when the testis differentiates, and meiosis does not begin until puberty. An understanding of the factors that regulate meiosis in germ cells could lead to applications for controlling fertility.

The resumption of meiosis in oocytes and follicular development is triggered by increased levels of the gonadotrophins, FSH and LH. Since oocytes lack receptors for gonadotrophins, these effects are due to actions on the surrounding cumulus granulosa cells, rather than directly on the oocytes. Gonadotrophins increase the production of cAMP in granulosa cells and induce synthesis of epidermal growth factor-like growth factors, meiosis-activating sterol (MAS) and gonadal steroid hormones, which are ultimately involved in the activation of MAPK (see Section 1.3). MAS is found in preovulatory follicular fluid and in adult testis and can activate meiosis in both male and female germ cells and across various species (humans, cattle, mice).

MAS has been identified as two steroids in the cholesterol biosynthesis pathway (Fig. 5.2), namely 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol and 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol. When oocytes surrounded by cumulus cells are treated with FSH, MAS is produced by the cumulus cells and acts in a paracrine manner to stimulate meiosis in the oocyte. The mechanism of how MAS stimulates the resumption of meiosis is not established; it may be that MAS is metabolized further to produce gonadal steroids, especially progesterone. For more information, see the review by Zhang *et al*. (2009).

Regulation of steroidogenesis

Primarily, LH and FSH regulate the production of steroid hormones by ovarian cells, although ST also plays an important role. ST may act by potentiating the effects of gonadotrophins through up-regulation of gonadotrophin receptors. ST increases the synthesis of some steroidogenic enzymes during the follicular and luteal phases of the oestrous cycle (see below) to increase the secretion of oestradiol and progesterone. ST increases the synthesis of steroid acute regulatory protein (StAR), which is responsible for translocating cholesterol into the adrenal mitochondria; this is the rate-liming step in gonadal steroidogenesis. ST also increases the synthesis of: cytochrome P450scc (CYP11A), which catalyses the side-chain cleavage step of cholesterol to form pregnenolone; CYP17A1, which converts pregnenolone and progesterone to androgens; and aromatase (CYP19A1), which converts testosterone to oestrogen (see Section 1.2). These effects are mediated in part by increased production of IGF-1 and in part by direct action of ST. ST can be produced by ovarian tissue, to act locally, as well as by the pituitary.

Reducing the enterohepatic circulation can decrease levels of steroid hormones. Steroid hormones are metabolized in the liver and eliminated in the bile as conjugates with sulfate or glucuronic acid (see Section 1.2). These conjugates can be

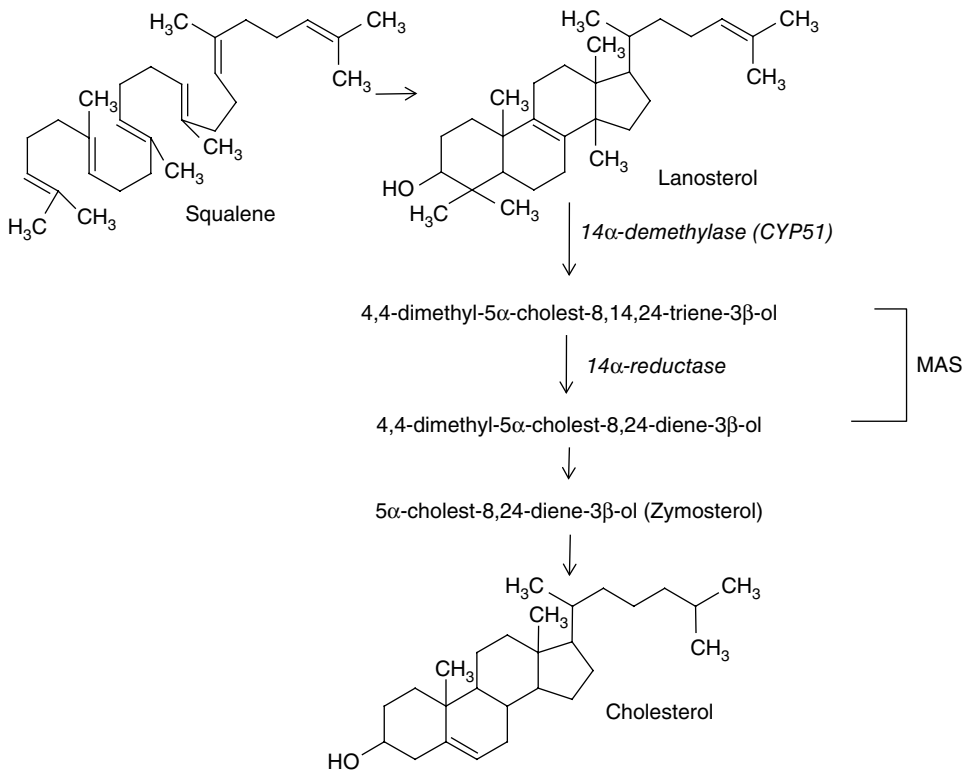


Fig. 5.2. Cholesterol biosynthesis pathway and formation of meiosis-activating sterol (MAS).

removed by bacterial metabolism in the intestine and the free steroids reabsorbed into the circulation. Increasing the liver metabolism of steroids, by treating with inducers of cytochrome P450, such as phenobarbital, or decreasing the absorption of steroids from the intestine, using binding agents or mineral oil, will increase the elimination of steroids and lower plasma steroid levels. For more information on steroidogenesis, see Sèdes *et al.* (2018).

Regulation of the oestrous cycle

In this section, the natural mechanisms regulating the oestrous cycle in mammals are presented. This information is expanded in the next section, which describes methods for manipulating the cycle.

Key concepts

- During the reproductive cycle in mammals, a group of ovarian follicles mature (follicular phase), the female becomes receptive to mating (oestrus or heat), and the dominant follicle ovulates, fertilization occurs and a corpus luteum (CL) is formed (luteal phase).
- GnRH produced by specific neurons in the hypothalamus drives the pulsatile release of LH and FSH from the anterior pituitary.
- GnIH neurons act on GnRH neurons in the POA to inhibit GnRH release, while kisspeptin has a stimulatory effect on GnRH neurons.
- Inhibin, activin and follistatin (activin-binding protein) are produced by the gonads. Inhibin reduces the production of FSH, while activin increases FSH production independently of GnRH.
- As the follicles mature, one or more dominant follicles acquire LH receptors and the other subordinate follicles undergo atresia, due to the lack of FSH.
- Oestradiol from the large follicles acts by positive feedback on the surge centre in the POA, increasing GnRH release to give a surge in LH release for ovulation.
- The corpus luteum develops rapidly from the follicle after ovulation and produces progesterone.
- If fertilization and implantation do not occur, high levels of progesterone and oxytocin from the ovary stimulate the uterus to secrete $PGF_{2\alpha}$, which drives luteolysis, and a new follicular phase begins.

Reproductive performance in dairy cattle has decreased with increasing milk production in dairy herds. Information on factors affecting the development of follicles, oestrous behaviour, ovulation and corpus luteum development and regression may be used to devise methods for improved reproductive performance. In dairy cattle, the goal is to manipulate the reproductive cycle to maximize milk production. In beef cattle, the goal is to maximize the number of calves produced by re-breeding the female within a set interval. The production of one calf per year for dairy and beef cows is considered optimal. Similar optimal goals for sows and ewes would be 30 piglets or 2 lambs per year, respectively.

Overview of the oestrous cycle

In the absence of pregnancy, the mature female undergoes a continuous series of reproductive cycles (Fig. 5.3), in which a group of ovarian follicles mature (follicular phase), the female becomes receptive to mating (oestrus or heat) and the dominant follicle ovulates, resulting in the release of an egg into the oviduct, allowing for fertilization to occur and a corpus luteum (CL) is formed (luteal phase). The number of follicles ovulated and CL formed is a characteristic of a species or sometimes a strain within a species. If fertilization of the egg and implantation of the embryo does not occur, the CL regresses (luteolysis) and the cycle repeats itself. The cycle lasts for approximately 21 days for cattle, pigs and goats, 19–25 days for horses and 16–17 days for sheep.

Follicular development

The maturation of ovarian follicles is driven by the gonadotrophic hormones, LH and FSH, which are released from the anterior pituitary (Fig. 5.4). The release of the gonadotrophic hormones is driven by gonadotrophin-releasing hormone (GnRH) and inhibited by gonadotrophin-inhibitory hormone (GnIH), which are produced by specific neurons in the hypothalamus. GnRH is normally released in a pulsatile manner from the 'tonic centre' in the median eminence and this release is under negative feedback control from gonadal steroids. GnRH neurons in the 'surge centre' in the POA respond by positive feedback to oestradiol and are responsible for the surge in production of GnRH at ovulation (see below).

GnIH is a member of the RFamide family of peptides (RFRP3) with an Arg-Phe-NH_2 motif at

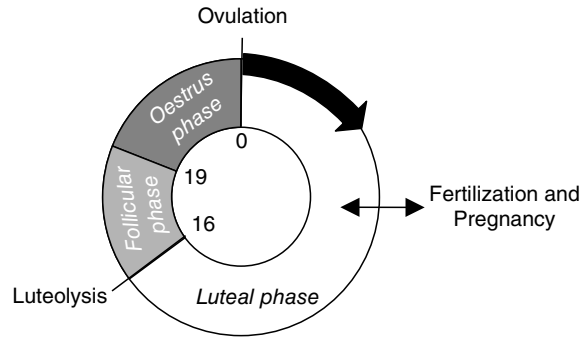


Fig. 5.3. Outline of the oestrous cycle.

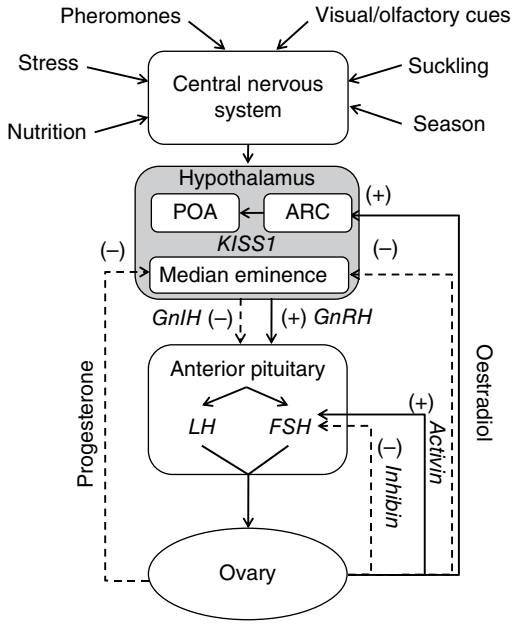


Fig. 5.4. Hormonal regulation of the oestrous cycle.

their C-terminal, which act as neurotransmitters and neuromodulators. GnIH neurons are localized in the hypothalamus, along with GnRH neurons, and they act on GnRH neurons in the POA to inhibit GnRH release and also on the pituitary to inhibit the release of gonadotrophins. Another RFamide neuropeptide, kisspeptin, (KISS1) has a stimulatory effect on GnRH neurons, leading to the release of GnRH. Kisspeptin is produced by KNDy (kisspeptin/neurokinin B/dynorphin) neurons, with neurokinin B activity stimulating kisspeptin release to initiate a GnRH pulse, while dynorphin inhibits kisspeptin release from the KNDy neurons to end GnRH pulses. GnRH neurons express the G protein-coupled receptor for kisspeptin (KISS1R, previously known as orphan receptor GPR54). The kisspeptin peptide exists in several forms, from 10 to 54 amino acids in length, with the biological activity in the last ten amino acids of the C terminal. Intravenous treatment with this 10 amino acid fragment increases plasma LH in sheep, horses, cattle, pigs and goats. Synthetic agonists of kisspeptin consisting of approximately five amino acids have been developed; the most potent agonist of the KISS1R is 4-(aminomethyl)-benzoic acid-3-(2-naphthyl) alanine-glycine-leucine-arginine-tryptophan-NH_2.

GnRH is delivered to the anterior pituitary via the hypothalamic–hypophyseal portal blood vessels (see Section 1.4). Both GnRH and the gonadotrophins are released in a pulsatile manner, with the pulse frequency and amplitude dramatically affecting biological activity. A high frequency of pulsatile GnRH release favours LH release, while low-frequency pulses favour FSH release. Low-frequency LH pulsatility is associated with follicular turnover and anoestrus, and moderate-frequency pulsatility is associated with ovulation. Many external factors affect the activity of the pulse generator in the central nervous system and thus affect the activity of the reproductive system. These factors are part of a 'basic rest–activity cycle' (BRAC) and include nutrition, sleep cycles, stress, suckling, presence of males, season, and visual and olfactory cues.

LH and FSH are glycoprotein hormones that each comprise two peptide chains: a common α-chain of 90 amino acids and a β-chain that is hormone

specific. Pig LH-β has 119 amino acids and pig FSH-β has 107 amino acids. The gonadotrophins have multiple roles, including controlling the development of ovarian follicles, ovulation, formation and function of the corpus luteum, and regulating the production of gonadal hormones. In the male, LH stimulates steroidogenesis in Leydig cells and FSH binds to Sertoli cells. Inhibin, activin and follistatin (activin-binding protein) are produced by the gonads and regulate the release of FSH by the pituitary. Inhibin and activin are members of the TGFβ family of polypeptides, which has more than 40 members, including myostatin and bone morphogenic proteins, which act via serine/threonine kinase receptors (see Section 1.3). Inhibition of these TGFβ superfamily members also results in potent anabolic and stimulatory effects in muscle, blood and bone, and a new class of molecule 'inhibitors of the activin receptor signalling pathway' (IASPs) have been developed. Inhibin and activin are both dimers, with the individual subunits linked by a single disulfide bond. Inhibin is a dimer of an alpha subunit and one of two different beta subunits, while activin is a dimer of the two beta subunits that form inhibin. Inhibin reduces the production of FSH (see Section 4.2), while activin increases FSH production independently of GnRH. Follistatins are a family of monomeric glycoproteins that bind to activin and prevent the stimulation of FSH production. For more information on activin and its receptor, see Lodberg (2021).

At birth, the mammalian ovary contains a large number of primary follicles (about 500,000 in cows), which gradually grow and mature as the animal matures. The number of primordial follicles (known as the ovarian reserve) is affected by developmental programming from environmental stress and the level of nutrition during fetal development or the peripubertal period. Primary follicles consist of an oocyte surrounded by a single layer of granulosa cells and interstitial tissue, which keeps the ovum suspended in the first stage of meiotic division. As the follicle grows, the oocyte increases in diameter and is surrounded by a zona pellucida. A fluid-filled cavity or antrum is formed and the layers of follicular cells lining the antrum increase. A second mass of highly vascularized steroidogenic theca cells develops as the theca interna and a group of non-steroidogenic cells form the theca externa around a basement membrane. During ovulation, the blood vessels rupture as the follicle collapses and the ovum is released, and the cells of the theca interna and granulosa are mixed. The granulosa and theca cells then differentiate into the large and small luteal cells of the CL, and the capillaries of the theca interna form the vascular supply of the CL.

Most follicles will undergo atresia after maturation, since only a limited number of ovulations occur. Over a 15-year period a cow can ovulate at most only 300 follicles, but allowing for ten pregnancies, this would be reduced to 60–80 ovulations or less. Growth of the follicles up to 4–6 mm in diameter in cows and 2–4 mm in diameter in sheep is independent of FSH, while FSH is required for further growth of the follicles. A group of follicles produce activin and are 'recruited' to begin maturation due to a transient increase in FSH. The size of this group can vary from 50 follicles in pigs to 5–10 in cattle and 1–4 in horses. The granulosa cells of the larger developing follicles produce oestradiol, using androgens that are produced by the theca cells. The granulosa cells of the large developing follicles also produce inhibin, while the other developing follicles produce follistatin, which together inhibit the release of FSH from the pituitary.

IGF-1 activity also increases as the follicles develop, due to decreased levels of IGF-binding proteins. IGF-1 may play a more important role in the growth of small follicles to recruitment, rather than in the growth of large follicles. Treatment with exogenous ST increases IGF-1 and the number of small follicles, and increases the number of CL in cattle and large follicles in pigs. ST acts both by increasing cell proliferation and by decreasing apoptosis and atresia of follicles. Expression of the *AMH* gene increases in granulosa cells to activate primordial follicles as the follicle transitions from primary through to early antral stages. *AMH* expression then declines through to the preovulatory stage as it limits the growth of activated follicles. Several different adipokines (see Section 3.5) also play significant paracrine or autocrine roles within the ovary, affecting steroidogenesis in granulosa cells, follicular development and atresia. For more information, see Kurowska *et al*. (2021).

As FSH levels decline, one follicle (or more in litter-bearing species such as pigs) acquires LH receptors on its granulosa cells and becomes the dominant follicle. Growth of this dominant follicle to a preovulatory follicle is dependent on pulses of LH. The growth of the other subordinate follicles is depressed, due to the lack of FSH via a potentiation of the production of oestradiol and inhibin by the

dominant follicle, and they undergo atresia. If there are insufficient pulses of LH, for example a mid-cycle increase in progesterone that adds to the negative feedback from oestradiol and inhibin, the dominant follicle also undergoes atresia, while a surge in LH results in its ovulation. A new wave of follicular growth (Fig. 5.5) occurs about every 10 days in cattle, so there will be two to three follicular waves during each oestrous cycle. Sheep have three to four waves, goats have four to five and horses have one to two waves in each cycle. Waves of follicular growth start before puberty and continue even throughout most of pregnancy, although during these times the follicles do not ovulate and become atretic, since high levels of progesterone during pregnancy block the positive feedback required for ovulation. In chickens and pigs, follicular growth is more continuous, it does not occur in waves and is not associated with any changes in plasma levels of FSH. Instead of one dominant follicle, many follicles develop to intermediate diameters, and follicles that are destined to ovulate are selected from this pool and continue to grow and ovulate. For more information on follicular development in farm animals, see the review by Juengel *et al*. (2021).

Oestrus and ovulation

For most of the ovarian cycle, oestrogen exerts negative feedback on GnRH release by the 'tonic centre' of the hypothalamus and on the sensitivity of the pituitary to LH release from GnRH. As the follicles mature, the dominant follicle secretes oestradiol, which acts on the brain to induce oestrous behaviour or 'heat'. Oestradiol also sensitizes the pituitary to GnRH binding and decreases the synaptic connections between GnRH neurons and inhibitory neurons. When levels of oestrogen are maximal, the positive feedback on GnRH neurons in the surge centre in the POA of the hypothalamus initiates a massive and transient release of GnRH from the POA neurons. GnRH then dramatically increases LH release by the pituitary, while negative feedback by oestrogen and inhibin reduces FSH release. This leads to a surge in LH release that culminates in rupture of the follicle and

For interest

Prolificacy in sheep is due to large genetic differences in ovulation rate among different breeds of sheep. Increased ovulation rates are due to mutations in single fecundity (Fec) genes encoding the TFG-β super family proteins in the Booroola (*BMPR1B*/*FecB*), Inverdale (*BMP15*/*FecX*) and Santa Ines breeds (*GDF9*/*FecG*). Increased ovulation rates in Lacaune sheep are due to a mutation in the beta-1,4-*N*-acetylgalactosaminyltransferase-2 (*B4GALNT2*/*FecL*) gene. In the Finn and Romanov breeds, increased ovulations are due to the effects of multiple genes which extend the period of follicular recruitment and allow follicles to be maintained from one wave to the next (reviewed in Juengel *et al*., 2018).

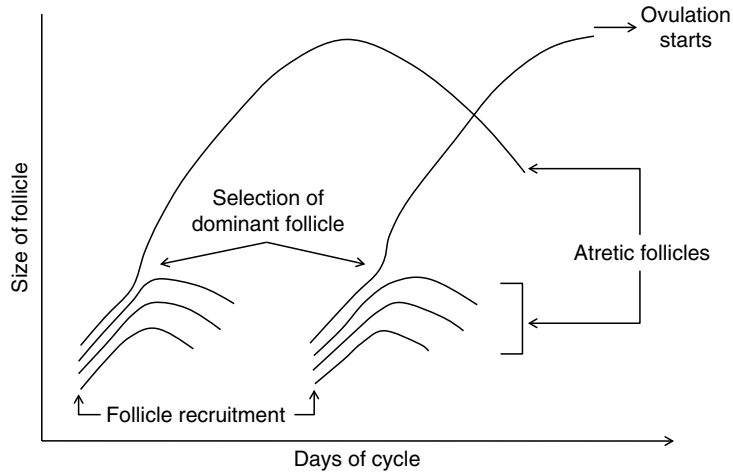


Fig. 5.5. Waves of follicular growth and development.

release of the ovum (ovulation) and formation of the CL on the ovary.

The positive feedback by oestrogen on the production of GnRH by the hypothalamus occurs only in females and involves oestrogen receptor α (ERα). However, GnRH neurons only express ERβ and not ERα, so the oestrogen positive-feedback pathway must involve other cells that express ERα. KNDy neurons in the arcuate nucleus (ARC) respond to increased oestrogen by increasing secretion of kisspeptin (KISS1), which stimulates adjacent GnRH neurons in the POA to release GnRH. Kisspeptin nerve fibres also make contact with GnRH neurons to directly regulate them. Levels of kisspeptin and its receptor KISS1R/GPR54 in the sexually dimorphic POA of the hypothalamus change throughout the oestrous cycle. They are both highest during the late follicular phase of the oestrous cycle at pro-oestrus, when the LH surge occurs due to positive feedback from oestrogen. KISS1 mRNA levels in the ARC are lowest when the cyclic pattern of oestrus is inhibited due to negative feedback of oestrogen on GnRH secretion. Kisspeptin may also directly influence LH secretion by gonadotrophs in the pituitary gland.

Kisspeptin neurons are also regulated by feed intake and nutritional status, season/photoperiod, pheromones, age and stress (see also Fig. 5.17). GnRH and kisspeptin neurons receive direct inputs from neuronal systems that are associated with metabolic status, such as orexigenic NPY and anorexigenic POMC neurons and orexin. The number of KISS1 mRNA-containing cells and GnRH neurons that receive input from kisspeptin are higher in the ARC during the breeding season than during the non-breeding season in ewes, goat does and mares. The number of kisspeptin neurons in the ARC increases in ewes transferred from a long-day (inhibitory) photoperiod to short days. Dopamine neurons repress GnRH in the non-breeding season. Thyroid hormone also affects the onset of the transition from polyoestrous to anoestrus. A number of environmental (heat, transport, isolation/restraint) and physiological (hypoglycaemia) stressors can inhibit reproduction, by reduced activation of the kisspeptin neurons to inhibit GnRH secretion. In sheep and goats, olfactory cues from pheromones from novel males can activate kisspeptin neurons, causing the 'male effect'. In the ewe, changes in kisspeptin expression are associated with puberty. For more information on the role of kisspeptin, see the review by Scott *et al.* (2019).

Luteal phase

The CL develops rapidly from the follicle after ovulation and is present during a large part of the oestrous cycle, known as the luteal phase. The CL is a transient endocrine structure that produces progesterone, which is required to prepare the uterus for implantation and for the maintenance of pregnancy. There are two types of steroidogenic cells in the CL: the large and small luteal cells, which are derived from the granulosa and theca cells of the follicle that ruptured at ovulation. LH stimulates the small luteal cells via the protein kinase A pathway to produce progesterone. The large luteal cells secrete high basal levels of progesterone, accounting for > 85% of the progesterone production by the CL, which is not very responsive to LH. These cells acquire receptors for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) later in the luteal phase (depending on the species), which can act via the protein kinase C (PKC) pathway to decrease progesterone production and induce apoptosis and cell death during luteal regression. The CL also contains many vascular endothelial and smooth muscle cells, which form blood vessels, as well as smaller numbers of pericytes, fibroblasts and immune cells. Intra-luteal substances produced by these cells also regulate progesterone production by the CL. The change in progesterone concentration throughout the luteal phase is also affected by blood flow in the CL. The CL grows rapidly and progesterone production increases at the beginning of the cycle (days 3–12 in the cow and days 2–8 in the ewe and sow) and then remains constant until days 15–16, when regression (luteolysis) begins to start a new cycle, unless there is a viable conceptus in the uterus. The presence of a functional CL during the luteal phase of the cycle prevents ovulation and any follicles that mature during the luteal phase will undergo atresia (except in the mare). Elevated levels of cortisol can also block or delay the preovulatory LH surge.

The development, maintenance and secretory functions of the CL are regulated by factors that are produced both within the CL and outside the ovary. In the newly developing CL, vascular endothelial growth factor (VEGF) is produced by the steroidogenic cells of the CL in response to hypoxic conditions to stimulate the growth of blood vessels. Nitric oxide (NO), which is a potent vasodilator and stimulates VEGF production, is produced by vascular endothelial cells. Basic fibroblast growth factor also stimulates proliferation of endothelial cells in the CL. Intra-luteal progesterone

is one of the most important factors in the maintenance of the CL. It is thought to act by stimulating its own production, as well as the production of prostaglandins and oxytocin, and by suppressing apoptosis of luteal cells.

Progesterone acts on the uterus to increase the number of receptors for oestrogen. If fertilization and implantation do not occur, oestradiol from the preovulatory follicle increases the number of receptors for oxytocin in the endometrium of the uterus. Binding of oxytocin to the oxytocin receptors stimulates the uterus to secrete $PGF_{2\alpha}$. $PGF_{2\alpha}$ travels locally to the CL and binds to receptors on the steroidogenic large luteal cells to inhibit the production of progesterone, initiate luteolysis and increase the release of oxytocin. $PGF_{2\alpha}$ up-regulates the expression of endothelin-1 and other vasoactive peptides, proinflammatory cytokines such as TNFα and IFN-γ, and NO by the CL, which act by autocrine/paracrine mechanisms to trigger the luteolytic cascade. Blood flow and progesterone production by the CL decrease, followed by the structural regression of the CL as cells undergo apoptosis. A new follicular phase then begins as new follicles mature to ovulation. For more information on luteal function and luteolysis, see the reviews by Abedel-Majed *et al*. (2019) and Teeli *et al*. (2019).

Manipulation of the oestrous cycle

Key concepts

- GnRH analogues, gonadotrophin preparations, synthetic steroids and antagonists, and prostaglandins are available for the manipulation of fertility.
- Chronic treatment with GnRH agonists causes an initial increase in LH and FSH, then down-regulation of GnRH receptors and inhibition of pulsatile LH release.
- Treatment of males with high levels of androgens reduces the secretion of LH and FSH by negative feedback on the pituitary.
- Oestrogens are luteolytic and are given before progestogen treatment to synchronize oestrus. Corticosteroids can be used for induction of parturition.
- Prostaglandin $F_{2\alpha}$ and its analogues are used for regression of the CL or to induce parturition.
- Anti-androgens inhibit the development of secondary sex glands and male sexual activity.
- Anti-progestogens are used to prevent implantation of the fertilized ovum, to cause resorption or abortion of established pregnancies or to hasten parturition at the end of pregnancy.
- Oestrus can be detected by behavioural observations and levels of progesterone in milk.
- Schedules for oestrus synchronization have been devised based on $PGF_{2\alpha}$, a combination of $PGF_{2\alpha}$ and GnRH, and a combination of progestins and $PGF_{2\alpha}$.
- Multiple ovulation and embryo transfer can be used to increase the number of valuable animals. Immature oocytes can be matured *in vitro*, fertilized, grown to the blastocyst stage and then transferred to recipient females.

During the normal reproductive cycle, a group of ovarian follicles matures during the follicular phase; the female becomes receptive to mating during oestrus; the dominant follicle ovulates; and a CL is formed during the luteal phase. The reproductive cycle can be manipulated in a number of ways to improve reproductive efficiency (Fig. 5.6). In the cycling female, luteolysis can be induced, oestrus and ovulation can be induced or regulated, and the number of follicles that ovulate can be increased. The pregnant female can be treated either to maintain pregnancy or to induce parturition. The non-cycling female can be treated to induce cycling, as in inducing puberty and ending seasonal anoestrus.

Hormone preparations for manipulating reproduction

Several hormone preparations are commercially available for the manipulation of fertility. These include GnRH analogues, gonadotrophin preparations, synthetic progestogens, oestrogens, corticosteroids and prostaglandins. In the USA, only GnRH and its analogues and $PGF_{2\alpha}$ and its analogues are approved for use in lactating dairy cows, although other hormonal treatments can be used in heifers and beef cattle.

Some of the commercially available GnRH agonists and antagonists are listed in Table 5.1 and gonadotrophin preparations used in veterinary medicine are listed in Table 5.2. Natural GnRH is a decapeptide, also known as gonadorelin. Potent analogues with higher receptor-binding affinities and longer half-lives include deslorelin and buserelin. Treatment with 2.5 μg of GnRH produces pulses

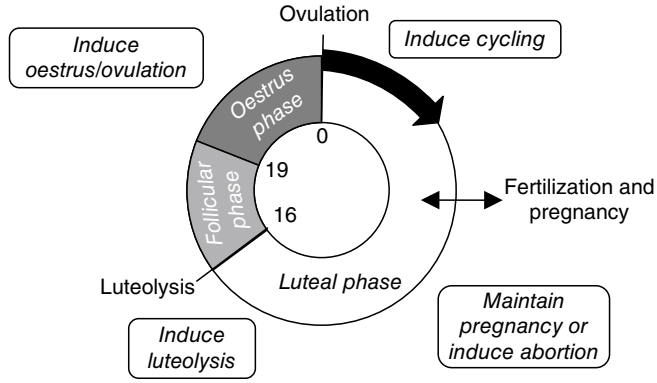


Fig. 5.6. Opportunities for manipulation of the oestrous cycle.

Table 5.1. GnRH agonists and antagonists.

Hormone	Sequence	Commercial name and company
GnRH agonists		
Natural GnRH	(pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH_2	Cystorelin® (Merial), Factrel® (Wyeth)
gonadorelin		and Fertagyl® (Intervet)
Deslorelin	([D-Trp^{6}, Pro^{9} NEt]-GnRH)	Ovuplant®, Suprelorin® (Peptech)
Buserelin	([D-Ser(tBu)^{6}, Pro^{9} NEt]-GnRH)	Suprefact® (Sanofi-Aventis)
Nafarelin	([pPyr^{1},D-2-Nal^{6}]-GnRH)	Synarel® (Pfizer).
GnRH antagonists		
Cetrorelix	([Ac-D-Nal^{1},D-Cpa^{2},D-Pal^{3},D-Cit^{6},D-Ala^{10}]-GnRH)	Cetrotide® (Aeternia Zentaris)
Ganirelix	([Ac-D-Nal^{1},D-Cpa^{2},D-Pal^{3},D-hArg(Et_2)^{6},hArg(Et_2)^{8},D-Ala^{10}]-GnRH)	Antagon® (Merck)
Nal-Glu-GnRH	([Ac-D-Nal^{1},D-Cpa^{2},D-Pal^{3},Arg^{5},D-Glu^{6}(AA), D-Ala^{10}]-GnRH)	NIH

Table 5.2. Gonadotrophin preparations.

Hormone	Biological name or activity	Commercial name and company
PMSG/eCG	Long-acting FSH activity, some LH activity	Equinex® (Ayerst) and Folligon® (Intervet)
FSH	Porcine pituitary FSH	FSH-P® (Schering) and Folltropin-V® (Vetrapharm), Ovagen® (ICP), NIH-FSH-S8®
hCG	LH activity	Chorulon® (Intervet)
LH	Porcine pituitary LH	Lutropin-V® (Vetrepharm)
PMSG and hCG combination		PG600® (Intervet)

of LH, while increasing the dose to 250 μg produces a surge in LH release that can cause ovulation of mature follicles. Other synthetic GnRH agonists and antagonists have also been developed for clinical use (Table 5.1). For more information, see the review by Millar *et al*. (2004).

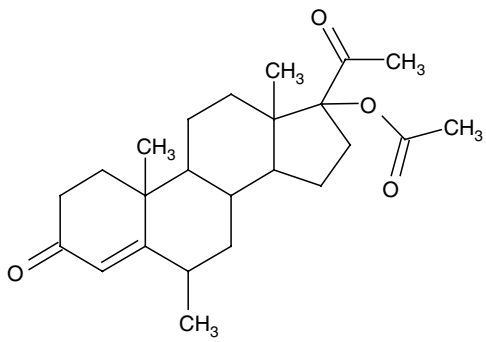
Pregnant mare serum gonadotrophin (PMSG), also known as equine chorionic gonadotrophin (eCG), has primarily long-acting FSH activity, but also some LH activity, and is used for oestrus induction and superovulation in cows and sheep. Several FSH preparations of pituitary origin are available. Human

chorionic gonadotrophin, isolated from the urine of pregnant women, has LH activity and is used to induce ovulation of mature follicles. LH from pituitary origin is also available. PG600 is a combination of PMSG and hCG that is used for puberty induction in gilts.

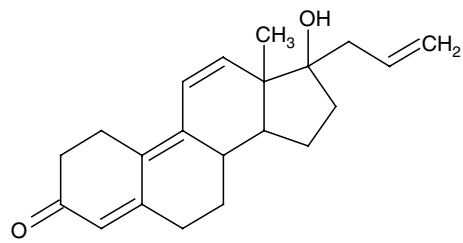
The commercially available progestogens, oestrogens and corticoids are summarized in Table 5.3 and the structures of orally active progestogens are given in Fig. 5.7. The main progestogen is progesterone, which primes the brain for oestrous behaviour after oestrogen exposure, suppresses the secretion of GnRH and is required for the maintenance of pregnancy. Progestogens are used in treatment programmes to induce or synchronize ovulation. Pretreatment with progestogens is necessary for

Table 5.3. Progestogens and anti-progestogens.

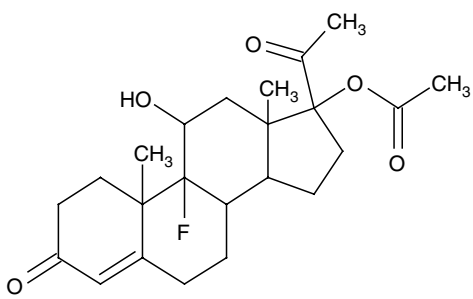
Category	Hormone	Biological name or activity	Commercial name and company
Progestogens	Melengestrol acetate (MGA), 6-methyl-17-acetoxy-progesterone (MAP), 6-chloro-8-dehydro-17-acetoxy-progesterone (CAP)	Orally active progestogens	MGA, MAP (Provera), CAP, Altrenogest® (Regu-Mate® from Hoechst), fluorogesterone acetate, Levonorgestrel®, Desogestrol®
	Norgestomet	Progestogen implant	Synchro-mate B® (Ceva laboratories)
Oestrogen	Oestradiol cypionate	Long-acting oestrogen	ECP (Upjohn)
Corticosteroids	Flumethasone, dexamethasone	Short-acting analogues	
	Dexamethasone trimethylacetate, triamcinolone acetonide	Long-acting analogues	



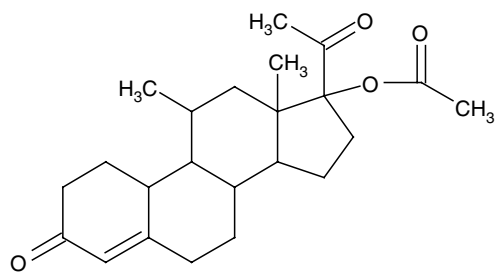
17-Acetoxy-6-methylprogesterone (MAP)



Altrenogest



Flurogestone acetate



Norgestomet

Fig. 5.7. Structures of some orally active progestogens.

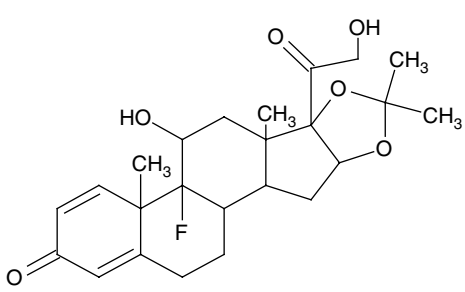
conditioning the behaviour centre of the hypothalamus to ensure oestrus in response to oestradiol, for the development of LH receptors in preovulatory follicles and for normal luteal function, after ovulation is induced with GnRH or LH.

Synthetic progestogens that can be given orally include 6-methyl-17-acetoxy-progesterone (MAP) and 6-chloro-8-dehydro-17-acetoxy-progesterone (CAP). Subcutaneous progesterone implants, progesterone-releasing intravaginal devices (PRIDs) and controlled internal drug-releasing devices (CIDRs) are popular methods to produce a sustained release of progesterone over a 7–10-day period to synchronize oestrus. Intravaginal sponges containing MAP or fluorogestone acetate (FGA) are used in ewes and Altrenogest® is used in mares to induce oestrus.

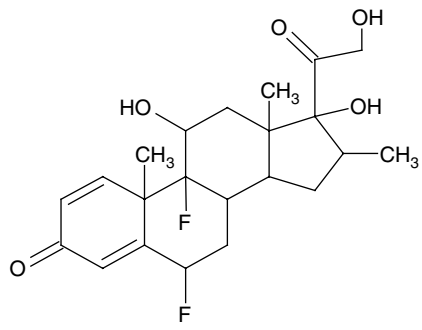
Oestrogens can be luteolytic, according to the status of the uterus and CL, and are often given before progestogen treatment in programmes to synchronize oestrus. Synthetic oestrogens and oestradiol esters (-cypionate, -benzoate, -valerate) are available that are more soluble and metabolized more slowly than oestradiol. Corticosteroids can be used for induction of parturition. Short-acting analogues include flumethasone and dexamethasone; and long-acting forms include dexamethasone trimethylacetate, triamcinolone acetonide and suspensions of flumethasone or betamethasone (Fig. 5.8).

Several steroid hormone antagonists have been developed (Table 5.4). Steroid hormone antagonists bind to the normal ligand-binding domain of a receptor, but they induce a different conformation of the receptor, which results in decreased hormone-dependent gene expression. Anti-progestogens (Fig. 5.9) include mifepristone, aglepristone and onapristone; the binding affinity varies with species and tissue type. The hydrophobic side chain at C17 is thought to be responsible for high-affinity binding to the receptor, while the additional side group at C11 causes a change in conformation of the receptor that inhibits the activity. Anti-progestogens can be used to prevent implantation of the embryo, to cause resorption or abortion of established pregnancies or to hasten parturition at the end of pregnancy.

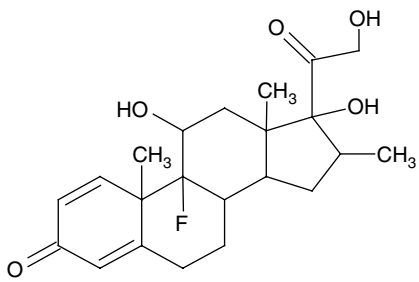
Anti-oestrogens and anti-androgens (Fig. 5.10) are used to treat hormonal-responsive cancers in humans, such as breast and prostate cancer. Tamoxifen and chlomiphene have mixed antagonist–agonist effects, depending on the species and tissue type. For example, in humans, tamoxifen acts as an anti-oestrogen



Triamcinolone acetonide



Flumethasone



Betamethasone

Fig. 5.8. Structures of some synthetic corticosteroids.

Table 5.4. Hormone antagonists.

Category	Hormone	Biological name or activity	Commercial name and company
Anti-progestogen	Aglepristone (RU46534) Onapristone (ZK98299)	Registered for veterinary use	Aglepristone® (Virbac), Schering AG
Oestrogen antagonists	Tamoxifen Clomiphene	Type I oestrogen antagonists with mixed antagonist–agonist effects	
		Type II anti-oestrogens with no agonist effects	ICI 164,384 ICI 182,780
Anti-androgens	Cyproterone acetate, flutamide		

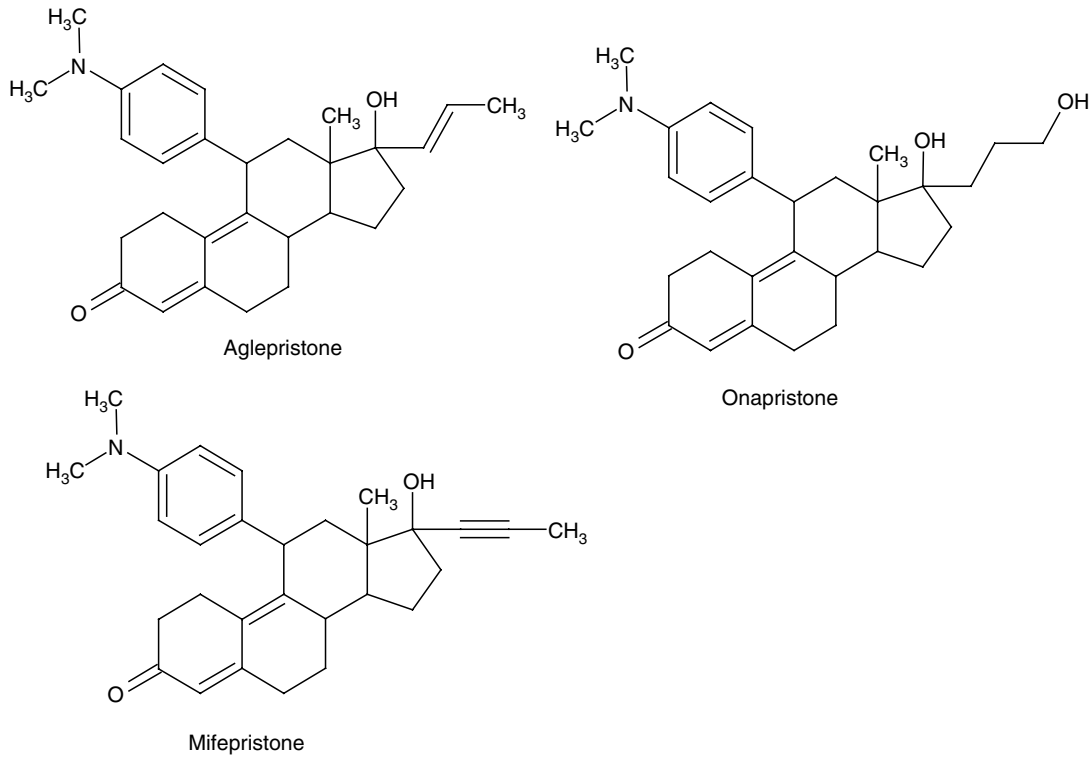


Fig. 5.9. Structures of some synthetic anti-progestogens.

in the mammary gland and an oestrogen agonist in the uterus. Type II anti-oestrogens include ICI 164,384 and ICI 182,780, which are 7α-alkyl-amide analogues and do not have oestrogen agonist activity.

Anti-androgens, such as cyproterone acetate and flutamide, inhibit the development of secondary sex glands in laboratory animals and male sexual activity in some species. Daily treatment with cyproterone acetate has been shown to prevent taint in boars (see Section 3.3).

Prostaglandin $F_{2\alpha}$ and its analogues (Table 5.5 and Fig. 5.11) are used for their luteolytic action (regression of the CL) or stimulation of the myometrium to induce parturition. This includes $PGF_{2\alpha}$ itself, available as dinoprost, as well as several synthetic prostaglandins. Synthetic prostaglandins are more potent luteolytic agents, which can be given at reduced dosages with fewer side effects, such as sweating and abdominal cramps, than $PGF_{2\alpha}$. Similarly, sows injected with $PGF_{2\alpha}$ to induce parturition have increased nest-building behaviour, while

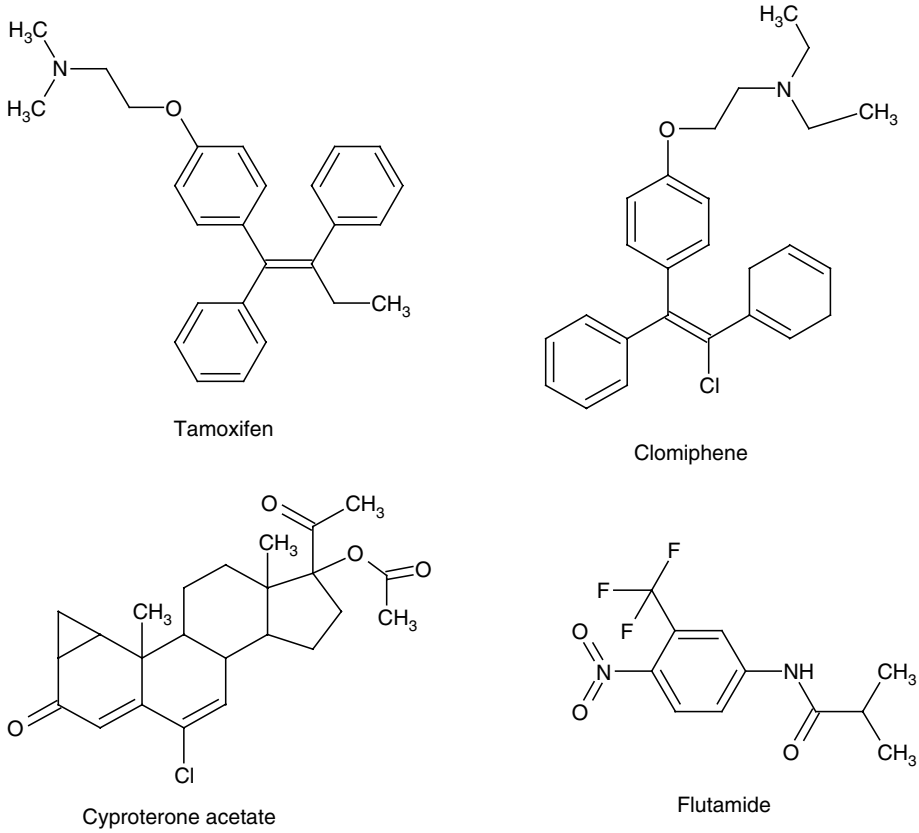


Fig. 5.10. Structures of some oestrogen and androgen antagonists.

Table 5.5. Prostaglandins.

Category	Hormone	Commercial name and company
$PGF_{2\alpha}$	Dinoprost	Lutalyse® (Upjohn)
Synthetic prostaglandins	Cloprostenol	Estrumate® (Mallicnkrodt)
	Fenprostalene	Bovilene® (Syntex)
	Alphaprostol	Alfavet® (Hoffman-La Roche)
	Fluprostenol	Equimate® (Miles)
	Prostalene	Synchrocept® (Syntex)

if cloprostenol is used to induce parturition, nest-building behaviour does not increase.

Use of hormone agonists to control fertility

GnRH analogues, progestins and androgens can act synergistically by negative feedback to reduce the release of gonadotrophins from the pituitary and thereby reduce fertility. The orally active progestogen MGA is used for suppressing oestrus and improving the rate of gain and feed efficiency in heifers. GnRH agonists (e.g. deslorelin, nafarelin) (see Table 5.1) have a higher affinity for GnRH receptors and a longer half-life in the circulation than GnRH itself. Chronic treatment with GnRH agonists initially causes a dramatic increase in the release of LH and FSH, followed by the down-regulation of GnRH receptors on pituitary gonadotroph cells and inhibition of pulsatile LH release. The lack of LH blocks ovulation but the ovary is still responsive to exogenous LH. Implants of GnRH agonists have been developed and can be

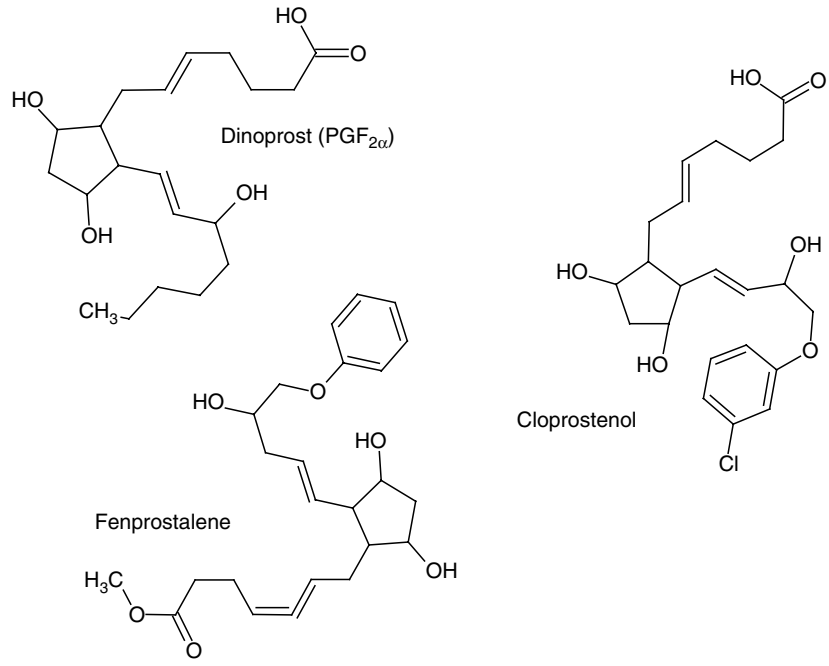


Fig. 5.11. Structures of some prostaglandin $F_{2\alpha}$ analogues.

used to prevent pregnancy in heifers under extensive management systems for up to 10 months. The procedure can also be used to control the time of ovulation by injection of LH in animals treated with GnRH agonist, which allows artificial insemination (AI) at a fixed time. This approach can be used to optimize ovulation rate and recovery of embryos in multiple ovulation and embryo-transfer protocols.

Bulls implanted with GnRH agonists have higher levels of testosterone for the duration of treatment. However, males of other species, such as the pig, have decreased levels of testosterone after treatment with potent GnRH agonists, which overstimulate the pituitary gland and shut down gonadotrophin release by a process termed homologous desensitization. GnRH antagonists (e.g. Nal-Glu-GnRH and Cetrorelix; see Table 5.1) have been used to reduce sperm counts in humans, but these peptides must be injected. They have the advantage of immediately suppressing the release of gonadotrophins and not inducing an initial burst in gonadotrophin release, which occurs with GnRH agonist treatment. Orally active non-peptide GnRH antagonists have also been developed and could be potentially used as contraceptive agents in livestock or captive animals.

Treatment of males with high levels of androgens reduces the secretion of LH and FSH by negative feedback on the pituitary, and thus androgens can act as contraceptive agents. For example, treatment of boars with testosterone propionate is reported to decrease the intensity of boar taint; and testosterone analogues are being investigated as male contraceptives in monkeys and humans. Testosterone itself is rapidly degraded by the liver and is thus not practical for use as a contraceptive agent. Testosterone esters, such as testosterone enanthate and testosterone undeconate, are effective in reducing sperm counts in humans. The 7α-methyl derivative of 19-nortestosterone, which cannot be reduced by the 5α-reductase enzyme, prevents dihydrotestosterone-like effects such as prostate hyperplasia. Combinations of testosterone esters and progestins (medroxyprogesterone acetate, Levonorgestrol® or Desogestrol®; see Table 5.3) are more effective contraceptive agents in humans. Implants of the progestins MGA or Levonorgestrol® are used to control fertility in zoo mammals. The use of a 'male pill' containing the anti-androgen cyproterone acetate with testosterone undeconate has also been studied in humans. For more information, see Adams (2005) and Herbert and Trigg (2005).

Transdermal patches can be used as an alternative to weekly injections or daily oral dosing for delivering combinations of androgens and progestins for contraceptive purposes but can result in skin irritation and are not used for commercial animals. Other complications from some steroid contraceptives include weight gain and a decrease in serum HDL-cholesterol.

Detection and synchronization of oestrus

METHODS FOR DETECTION OF OESTRUS During oestrus or 'heat', the female will accept a male; and during 'standing heat' she will stand to be mounted. This behaviour is related to increased levels of oestradiol and decreased levels of progesterone following ovulation. In pigs, this lordosis can be more easily detected by pushing forward on the rear of the sow. The efficiency of detection of oestrus in cattle can be less than 50%, due to short and weakened oestrous signals, and this is a major cause of reproductive failure. Oestrous behaviour is affected by environmental factors such as temperature, season and light, age and body weight, hormonal imbalance, diet and amount of production. Either methods to improve heat detection or strategies to control the time of ovulation can be used to improve conception rates. The optimal insemination time is a few hours before ovulation, which happens about 24–38 h after standing heat begins.

OESTROUS BEHAVIOUR Cows need adequate space to display mounting behaviour, with softer footing such as grass, dirt or straw. Mounting behaviour is decreased on concrete floors or floors that are too slippery or too uneven. Cows with sore feet or legs have less mounting activity. Cows that are themselves in heat or have recently been in heat are most likely to mount a cow in heat. As the number of cows in heat increases up to three to four, the number of mounts per cow increases dramatically.

The extent of mounting activity can be used as an indicator of oestrus. The tail-head area can be scarred and dirty from mounting by herd mates. An older but effective technology is to use a brightly coloured enamel-based paint on the tail-head and cover the paint strip with a contrasting colour of chalk. The extent of cover of tail paint and chalk is scored using a scale of 5 (for no signs of oestrus and full presence of paint and chalk) to 0 (for standing oestrus and absence of paint and chalk). Pressure-activated heat-mount detectors that change colour from the weight of the mounting animal can also be fixed to the tail-head. There are more sophisticated pressure sensors available, which either display the frequency of mounting behaviour directly or send signals to a remote device that records the mounting behaviour. Vasectomized teaser bulls, which can be fitted with a chin-ball marking harness, are particularly useful in detecting heat when there are only a few cows in heat. Cows or heifers treated with testosterone or oestradiol can also be used as an alternative to vasectomized bulls.

Standing heat can often be brief and is not always observed, since it occurs mostly in the early morning and late evening, but various secondary signs can be used to detect oestrus. During oestrus, vaginal mucus that is clear and stringy is discharged and the vulva is reddened, but this can be difficult to detect in cows. Blood stains on the tail or vulva are indicative of a recent heat. Cows in heat are restless, bellowing and trailing other cows. Electronic pedometers fixed to the leg of the cow can be used to measure an increase in walking activity as a measure of oestrus. There is increasing emphasis on monitoring physical activity as an indicator of oestrus, since oestrous cows are more active than normal. Feed intake and milk yield can also decrease during oestrus. Changes in perineal odours occur near oestrus and these can be detected using trained sniffer dogs or potentially using an 'electronic nose', which has electronic sensors that change electrical characteristics when exposed to volatile compounds. All these changes are driven by the increase in oestradiol and decrease in progesterone.

Follicular development can be estimated by palpating the ovaries via the rectum or using real-time ultrasound equipment with a transducer placed in the rectum. Pregnancy can be confirmed by this type of examination at about 40 days after breeding. Measurements of electrical impedance have also been suggested to predict the time of ovulation and parturition, as well as for detecting ovarian follicular cysts and endometriosis. However, the physiological basis for these observations has not been well established. For more information, see the review by Řezáč (2008).

MILK PROGESTERONE The level of progesterone in milk can be used to evaluate oestrus and pregnancy in dairy cattle. The level of progesterone rises slowly for the first 4–6 days after ovulation and reaches a maximum at days 10–17. It falls sharply at days 18–19 in non-pregnant cows, due to luteolysis,

but remains elevated in pregnant cows, since the CL continues to function.

The accuracy of pregnancy diagnosis using milk progesterone is only about 80%, due to factors such as errors in oestrus detection, differences in cycle length, uterine disease, ovarian cysts and early embryonic mortality. It can be used more reliably to determine if a cow is not pregnant. This involves comparing progesterone levels in milk samples taken at the time of insemination to progesterone levels in milk at 21–24 days later.

A high level of progesterone in milk can confirm the lack of oestrus and that a cow should not be inseminated. This can be particularly useful in high-producing cows or cows under heat stress when there are poor outward signs of oestrus. A low progesterone level suggests that the cow might be near oestrus but does not confirm that it is at the optimum stage for insemination. Low progesterone levels can also be due to inactive ovaries or the presence of follicular cysts. Luteal cysts can be distinguished from follicular cysts by a high level of progesterone. The presence of a functional CL can be confirmed by accompanying high progesterone levels in cows that will be used as embryo-transfer recipients.

Milk progesterone can be evaluated using commercially available kits for on-farm use. Progesterone could potentially be measured during milking with an online system.

STRATEGIES FOR SYNCHRONIZING OESTRUS

Synchronization of oestrus involves manipulating the ovarian cycle to mate the female at a predetermined time. This allows for greater management control of reproduction, improved reproductive efficiency and a predetermined parturition time. Synchronizing oestrus reduces the number of checks required for determining oestrus and increases the intensity of oestrus when a group of cows are in oestrus at the same time. Timed AI can also be used on all cows at the same time when protocols designed to bring all cows into oestrus within a narrow window of time are used. Oestrus synchronization is also necessary for embryo-transfer programmes.

Effective methods for the synchronization of oestrus are also necessary for AI to be used in the beef cattle industry, although AI is used to a much smaller extent in beef cattle than in dairy cattle. This is because of the extensive nature of beef cattle operations, while dairying is an intensive operation. Increased use of AI would allow the use of superior genetics in the beef industry, as has been done in the dairy industry for some time. Fertility is the most important trait for beef cattle, since beef breeders get most of their income from calves born into the herd.

The fertility of the first oestrus after parturition ('foal-heat' in mares) is low and the timing of ovulation can be erratic, so animals can be treated with luteolytic agents at this time to induce regular oestrus. Oestrogen (as oestradiol cypionate; see Table 5.3) is also used to induce oestrus in 'jump' mares used as teasers for semen collection from stallions.

Oestrus synchronization includes a method for controlling follicular wave development, promoting ovulation in anoestrous cows, regressing the CL in cycling cows and synchronizing oestrus or ovulation at the end of the treatment. These methods must produce a high proportion of females in heat at a predetermined time, maintain high rates of fertility and have no undesirable side effects. In addition, they should be easy to perform and be of low cost.

The presence of a functional CL during the luteal phase of the cycle prevents ovulation. Progesterone produced by the CL delays the maturation of LH-dependent follicles and primes the brain for oestrous behaviour. In the absence of pregnancy, $PGF_{2\alpha}$ causes luteolysis and decreases progesterone levels. This restores the LH pulse frequency and amplitude, allowing the subsequent development of the dominant follicle, leading to oestrus, ovulation and normal luteal function. Treatment of animals having a functional CL with $PGF_{2\alpha}$ will thus result in oestrus and ovulation a few days thereafter.

Schedules for oestrus synchronization have been devised based on $PGF_{2\alpha}$, a combination of $PGF_{2\alpha}$ and GnRH, and a combination of progestins and $PGF_{2\alpha}$.

PROSTAGLANDIN $F_{2\alpha}$-BASED SYSTEMS

Injection of $PGF_{2\alpha}$ will induce regression of the CL and not adversely affect subsequent oestrous cycles. However, $PGF_{2\alpha}$ is not effective on newly established CL, and cows injected on days 1–5 of the oestrous cycle are non-responsive. There are several waves of follicular development in each oestrous cycle and cows injected on days 7 or 15 of the oestrous cycle have a highly developed follicle ready to ovulate after CL regression. $PGF_{2\alpha}$ treatment on days 7 or 15 will therefore induce oestrus 3 days after injection. A practical approach (Fig. 5.12) is to inject $PGF_{2\alpha}$ twice, 14 days apart, so that the cows

will be responsive to $PGF_{2\alpha}$ at least at the second injection and come into oestrus shortly thereafter. Alternatively, cows can be injected at the beginning of the week and those that come into oestrus later in the week are inseminated. The remaining cows are injected with $PGF_{2\alpha}$ at 14 days after the first injection and then inseminated when oestrus is detected or, at the latest, 4 days after the last injection of $PGF_{2\alpha}$. However, not all cows respond to $PGF_{2\alpha}$ treatment, particularly before day 12 of the oestrous cycle. Improved synchronization of oestrus is obtained with an additional injection of $PGF_{2\alpha}$ 2 weeks before the initial injection. Cows are re-bred 21 days after the first insemination if they are seen to be in oestrus. This decreases the number of days open and the net cost per cow but this process requires detection of oestrus, keeping accurate records and the identification of individual animals.

GnRH and the Ovsynch® protocol Treatment with $PGF_{2\alpha}$ results in regression of the CL but does not synchronize the growth of follicles or affect the preovulatory surge of LH. Injection of GnRH at 7 days before treatment with $PGF_{2\alpha}$ synchronizes follicular growth and oestradiol secretion with luteolysis and improves the timing of oestrous behaviour. The Ovsynch® protocol (Fig. 5.13) utilizes GnRH and $PGF_{2\alpha}$ in a procedure that allows for AI without oestrus detection in lactating dairy cows. The protocol can be used at any point in the oestrous cycle and is particularly useful when the efficiency of oestrus detection is poor. The Ovsynch® protocol without oestrus detection results in pregnancy rates that are similar to multiple treatments with $PGF_{2\alpha}$ every 14 days and insemination at detection of oestrus. In addition, the Ovsynch® protocol reduces the number of days postpartum to first insemination and the number of days open.

The initial treatment with GnRH will ovulate a dominant follicle if it is present and initiate a new follicular wave, or a new follicular wave may be occurring spontaneously. The injection of $PGF_{2\alpha}$ given 7 days later will cause regression of the CL. A second injection of GnRH is given 48 h later and will cause the dominant follicle that has grown from the first injection of GnRH to ovulate. Cows are inseminated 12–16 h after the second GnRH injection so that capacitated sperm are present at the time of ovulation. In some cases, cows will be in oestrus shortly after treatment with $PGF_{2\alpha}$ and these cows should be inseminated at that time. Cows can also be inseminated at the time of the second GnRH treatment. This modification of the Ovsynch protocol is known as Cosynch and is commonly used with beef cattle because it requires less animal handling.

The Ovsynch® protocol does not improve conception rates in heifers compared with insemination at detected oestrus. However, the Ovsynch® protocol does remove the difficulty of detecting oestrus and therefore reduces the number of days open in heat-stressed cows. It is also useful in treating cows with either ovarian or follicular cysts without identifying the type of cyst, since the GnRH treatment

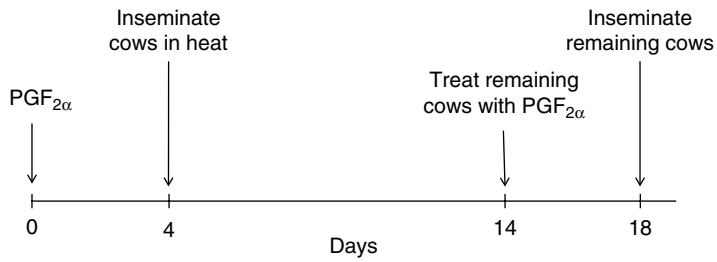


Fig. 5.12. Prostaglandin-based system of oestrus synchronization.

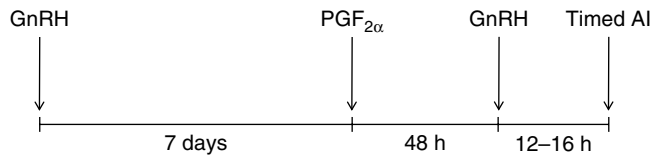


Fig. 5.13. Ovsynch® protocol for oestrus synchronization.

will remove the follicular cyst and $PGF_{2\alpha}$ treatment will remove the luteal cyst (see below). Cows with inactive ovaries in which follicular development has ceased do not respond well.

Presynch-Ovsynch, a modification of the Ovsynch® protocol, utilizes two injections of $PGF_{2\alpha}$ 14 days apart, with the second injection given 12 days prior to the first injection of GnRH. This pre-synchronization step increases the probability of having a dominant follicle ready to ovulate with the first GnRH injection of the Ovsynch® protocol and improves the pregnancy rate from 25% to 43% in lactating cycling dairy cows. In a further modification, the second GnRH injection is moved from 48 h to 72 h after $PGF_{2\alpha}$ injection and cows are inseminated at the same time as the GnRH injection. This strategy allows for an extended pro-oestrus period, which results in a more mature functional follicle that ovulates, and this significantly improves pregnancy rates. Another modification that results in higher pregnancy rate compared with Presynch is Double Ovsynch, which involves two Ovsynch protocols seven days apart, followed by insemination. Injection of bovine somatotrophin (see Section 3.4), either at the first GnRH injection or at the time of insemination, improves pregnancy rates and embryo growth and survival.

PROGESTIN-BASED SYSTEMS A modification of the Ovsynch® protocol that includes treatment with a progestin for 7 days before treatment with $PGF_{2\alpha}$ prevents the early ovulation and improves the synchronization and detection of oestrus and conception rates. Short-term exposure to progestins will also induce the onset of oestrous cycles in a proportion of anoestrous cows and heifers. Early use of progestins for oestrus synchronization utilized treatments in excess of 14 days. These resulted in abnormal follicular growth and decreased fertility and were linked to deleterious effects on sperm transport and viability in the reproductive tract of ewes. Short-term treatment of progestin of less than 10 days' duration is now used along with a treatment to cause regression of the CL, such as $PGF_{2\alpha}$ or oestrogen (Fig. 5.14).

Progestin is normally administered to dairy cows using implants (CIDR or PRID). For beef cattle, norgestomet is available in the Synchro-Mate-B® implant or MGA is given in the feed (see Table 5.3). The implant is placed at the base of the ear and an injection of oestradiol valerate and norgestomet is given when the implant is inserted. The CL regresses spontaneously while the implant is present. The injection of oestrogen and progestin is designed to cause early regression of the CL in cows in the early stage of the cycle. Alternatively, MGA can be fed to beef cattle for 14 days, followed 17 days later by an injection of $PGF_{2\alpha}$ (Fig. 5.14). The treatment with MGA will initiate oestrus in anoestrous females and synchronize oestrus in cycling females but will result in a low-fertility oestrus in the 7 days after MGA is withdrawn. The $PGF_{2\alpha}$ treatment occurs in the latter part of the next oestrous cycle, which will cause a high rate of CL regression and synchronization of oestrus. This procedure induces oestrus in about 80% of yearling heifers and is used for this purpose. Since the protocol takes about 34 days to achieve synchronized oestrus, it cannot be used with postpartum cows that calved late in the season without affecting the start of the new breeding season.

Several protocols have been developed using progestins to induce puberty and synchronize oestrus in beef heifers. A progestin treatment (norgestomet implant) can be used between the GnRH treatment and the $PGF_{2\alpha}$ treatment (GnRH–P–PGF). A further modification of an additional GnRH treatment 48 h

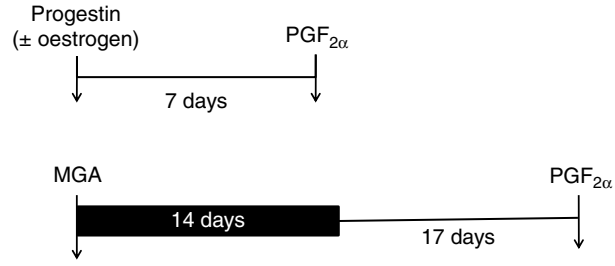


Fig. 5.14. Progestin-based protocols for oestrus synchronization.

after the $PGF_{2\alpha}$ treatment (GnRH–P–PGF–GnRH) induces a final LH surge and a highly synchronous time of oestrus, which allows the use of timed AI without oestrus detection. Another approach uses an initial injection of oestrogen and a progestin implant (CIDR) for 7–8 days, followed by an injection of $PGF_{2\alpha}$ when the CIDR is removed and a second injection of oestrogen 24–48 h later (E–CIDR–PGF–E). The first oestrogen injection will cause regression of FSH-dependent follicles and the CIDR implant with progestin induces turnover of LH-dependent follicles. Once these follicles become atretic, a new wave of follicular development begins. The injection of $PGF_{2\alpha}$ will cause any CL present to regress, and the final oestrogen injection will induce oestrus and ovulation. The induction of puberty and oestrus is more effective the closer the animals are to a naturally occurring oestrus.

Controlled breeding programmes result in significant financial savings to dairy producers. The additional costs for drugs and labour are more than offset by the savings from decreased labour for oestrus detection, lower costs from fewer days open and fewer replacement heifers needed. Producers can also control the time of calving, to take advantage of seasonal variations in pricing and constraints to production. Beef cattle producers can use controlled breeding programmes to induce puberty and synchronize oestrus in heifers by the start of the breeding season, so they will calve early and improve lifetime production. For more information on the manipulation of the oestrous cycle in dairy and beef cattle, see the reviews by Colazo and Mapletoft (2014) and Nowicki *et al.* (2017).

Synchronization of oestrus in pigs can be accomplished by treatment with progestogen for 14–18 days (PG600; see Table 5.3) and then breeding at oestrus. Ewes during the breeding season can be injected with $PGF_{2\alpha}$ twice 9 days apart and then treated with an intravaginal sponge or a small CIDR impregnated with MAP (see Table 5.3) for 12 days. Ewes will exhibit oestrus 48–72 h after the removal of the sponge. In the non-breeding season, PMSG treatment (see Table 5.2) is also necessary at the end of the progesterone regimen to induce ovulation.

Superovulation and embryo transfer

Several *in vitro* techniques can be used to improve reproductive efficiency and increase the contribution of valuable females to the gene pool. Large numbers of oocytes or embryos can be collected from female donors and transferred to recipient females. Immature oocytes can be matured *in vitro*, fertilized, grown to the blastocyst stage and then transferred to recipient females.

Multiple ovulation and embryo transfer (MOET) involves the recovery of multiple embryos after superovulation and transfer of the embryos to synchronized recipients of lesser genetic merit. To induce superovulation, FSH or PMSG is given in the middle of the cycle (days 8–14 in cattle) to increase the number of follicles that mature into dominant follicles and to reduce the regression of follicles. A single injection of PMSG (which has a longer half-life than FSH), or twice-daily injections of FSH for 4 days, is required. This is followed by treatment with $PGF_{2\alpha}$ on day 3 to induce luteolysis, and then twice-daily FSH injections are continued to induce superovulation until day 4, when oestrus occurs. Twice-daily inseminations are given, starting 12 h from the start of oestrus. Sperm transport can be reduced by superovulation, particularly when PMSG is used, so inseminations directly into the uterus can be used to improve fertility. Embryos are collected by flushing the uterus non-surgically at 6–8 days after oestrus in cattle and horses, but surgical recovery is used for pigs, sheep and goats. Embryos are assessed for the stage of development and for evidence of physical damage; and good-quality embryos can then be transferred to recipients or frozen. Recipient animals should be checked for the presence of a CL, since a CL is required to maintain the pregnancy after transfer of the embryos. The Ovsynch® protocol or other methods of oestrous cycle control can be used to synchronize ovulation in recipient cows and improve the success rate of embryo transfers. For more information, see the review by Jones and Lamb (2008).

The ovulation rate and number of transferable embryos can be improved by treatment with anti-PMSG serum at oestrus or after the LH surge has occurred. This suppresses the second wave of follicular development and reduces the number of follicular cysts. A low LH content in the FSH preparation is desirable, since LH interferes with follicular development. The presence of a large dominant follicle reduces the number of follicles that will mature and ovulate in response to exogenous gonadotrophins. There is also the possibility that the animals will produce antibodies against the non-homologous gonadotrophins that are used for superovulation, so that later treatments with gonadotrophins will not be successful.

***IN VITRO* PRODUCTION OF EMBRYOS** Large numbers of embryos can also potentially be produced from immature oocytes that have not yet committed to atresia. The pig ovary has over 200,000 primordial follicles, while the cow has about half that number. Oocytes can be collected at slaughter or by aspiration of follicles from both mature and immature females using ultrasound-guided ovum pickup (OPU). This allows the production of embryos from donors with poor fertility or those that do not respond to superovulation. This is particularly useful for the recovery of genetic material from slaughtered animals. It also has potential for the conservation of rare breeds. Oocytes can also be recovered from young prepubertal females, thus shortening the generation time and increasing the number of potential offspring from valuable individuals. After recovery, the oocytes are matured and fertilized *in vitro* and grown to the blastocyst stage before freezing or transfer to a recipient animal.

The efficiency of blastocyst production from *in vitro* production systems is highly variable and lower than with oocytes recovered after insemination. Blastocyst production depends on the quality of the gametes used and human technical skills. Culture conditions are also important and the optimum culture conditions vary among different species. Good-quality oocytes that are surrounded by intact cumulus cells and have a homogeneous-looking cytoplasm should be used. *In vitro* maturation (IVM) of oocytes includes both nuclear maturation, seen as the development of a metaphase II spindle, and cytoplasmic maturation, which allows the formation of a male pronucleus after fertilization. IVM has been performed using several different systems, including co-culturing the oocytes with oviductal epithelial cells or granulosa cells, or supplementation of media with follicular or oviductal fluid. Defined culture media have also been used and are preferred, since they reduce the potential for contamination with bacteria and viruses from cells in co-culture and reduce abnormal embryonic growth leading to 'large offspring syndrome' in sheep and cattle. Adding gonadotrophins (LH and FSH) along with EGF or IGF-1 to the maturation medium can stimulate oocyte maturation in defined media. One-third of porcine oocytes that are treated with EGF (10 mg ml^{-1}) develop to the blastocyst stage after *in vitro* fertilization (IVF).

A major problem with IVF of porcine oocytes is polyspermy, which occurs when several sperm penetrate each oocyte and the oocyte then fails to develop properly past the blastocyst stage. This is rarely observed with *in vivo* insemination prior to ovulation, since there are a limited number of sperm arriving simultaneously at the oocyte and the zona pellucida surrounding the oocyte effectively blocks the penetration of more than one sperm. The incidence of polyspermy has been overcome, in part, by improving the quality of the IVM oocytes. The success of fertilization is seen ultimately in cleavage of the oocyte. Both fresh and frozen–thawed sperm have been used for IVF in cattle and swine. Sperm cells can be sorted into separate pools of X- and Y-bearing sperm using flow cytometry and used to produce offspring of the desired sex. Alternatively, a few cells can be removed from an embryo and the sex can be determined using X- and Y-specific DNA probes with PCR.

Embryos are a convenient and cost-effective form for providing new genetics to animal production systems and to increase genetic gain and progress using elite individuals as donors. Since embryos can be produced that are free of disease, they are especially suitable for international trade. Embryo recovery and transfer can also be used to eliminate disease by repopulating herds with uninfected animals. Embryos that are recovered from infected animals can be treated with trypsin and washed to remove bacteria and viruses so that they are free of the disease, since the zona pellucida protects the embryo from infection. The embryos are then transferred to clean individuals to produce uninfected animals at birth. For more information on embryo production and transfer in farm animals, see the reviews by Ferré *et al*. (2020) and Ealy *et al*. (2019).

Pregnancy, parturition and the postpartum interval

Key concepts

- Maternal recognition of pregnancy involves signalling from the conceptus to maintain luteal function or prevent luteolysis.
- High levels of progesterone and oestrogen reduce GnRH release from the hypothalamus and LH release by the pituitary during pregnancy.
- Supplementing with exogenous progesterone, development of an accessory CL and delaying the postpartum oestrus may be useful in maintaining pregnancy.

- Parturition is triggered by corticosteroids produced by the fetus in response to CRH released by the fetus and placenta.
- Corticosteroids stimulate the production of oestrogens by the placenta, which stimulates the production of $PGF_{2\alpha}$ and increases the number of oxytocin receptors in the uterus. Oxytocin released when the fetus enters the birth canal increases the production of $PGF_{2\alpha}$ and both hormones increase contractions in the uterus.
- Parturition can be induced with combinations of corticosteroids, $PGF_{2\alpha}$ and oxytocin.
- Parturition can be delayed using CRH receptor antagonists or inhibitors of prostaglandin synthase.
- There is normally a period of anoestrus due to decreased LH production before the first oestrus occurs postpartum.
- Increased frequency of milking decreases the sensitivity of the hypothalamus to positive feedback from oestrogen to delay ovulation.
- Ovarian cysts can be treated by injection of GnRH. Luteal cysts that produce high levels of progesterone can be treated by injection with $PGF_{2\alpha}$.
- Stress and a reduced energy balance can increase the period of postpartum anoestrus. Leptin may signal the reproductive system that sufficient body fat is present to support a pregnancy.

Recognition and maintenance of pregnancy

Following fertilization in the oviduct, the embryos enter the uterus, develop into spherical blastocysts and hatch from the zona pellucida. The blastocysts then expand and may transition to a tubular and filamentous form to establish a large surface area for nutrient and gas exchange. The blastocysts orient to the epithelial cells of the endometrium to initiate pregnancy recognition, followed by adhesion of the blastocyst to the epithelial cells within a narrow window of time for implantation, depending on the species. In pigs and horses, the cells of the trophectoderm of the embryo and the epithelial cells of the uterus remain in close contact throughout the pregnancy, while in ruminants the trophectoderm cells mix with the uterine epithelial cells. In carnivores, rodents and primates, the blastocysts invade the uterine vasculature to achieve close contact to the maternal blood.

A functional CL is necessary for the maintenance of pregnancy in all farm animals. Maternal recognition of pregnancy involves signalling from the conceptus to either act directly on the CL to maintain luteal function, or inhibit the release of $PGF_{2\alpha}$ from the uterus and prevent luteolysis. In sheep, cattle and goats, the embryonic pregnancy recognition signal is an anti-luteolytic protein produced by the conceptus. This was named trophoblast protein-1 (TP-1) and later renamed interferon-τ (IFN-τ), due to the sequence similarity with type-I interferon. IFN-τ acts on the endometrium in a paracrine manner to prevent luteolysis by decreasing the number of oxytocin receptors in the uterus. This decreases the production of $PGF_{2\alpha}$ by the uterus, which suppresses luteolysis, so that the CL is maintained and continues to produce progesterone. IFN-τ also protects the conceptus and uterus against viral infection and modulates the maternal immune response to avoid rejection of the embryo.

Maternal recognition of pregnancy in pigs is driven by oestrogen produced by the trophectoderm cells of the embryo, which changes the endocrine secretion of $PGF_{2\alpha}$ by the uterus to exocrine release of $PGF_{2\alpha}$ into the uterine lumen, where it is metabolized to prevent luteolysis. $PGF_{2\alpha}$ also modulates the expression of uterine genes involved in implantation and may cause local contraction to distribute the embryos within the available space in the uterus. At least one embryo in each of the uterine horns is required to prevent local production of $PGF_{2\alpha}$ and prevent embryo loss. The placenta secretes increasing amounts of oestrogens during the second and third trimesters. High levels of progesterone and oestrogen reduce GnRH release from the hypothalamus and LH release by the pituitary.

In the mare, the decreased production of $PGF_{2\alpha}$ by the uterus is also essential to maintain CL function to support pregnancy. $PGF_{2\alpha}$ production is decreased by the presence of the embryo, which secretes several different products during early pregnancy, including steroids, prostaglandins and different proteins and peptides. The embryo also moves throughout the uterine lumen before implantation and this migration may produce a mechanical signal that prolongs luteal function. However, the mechanism of the maternal recognition of pregnancy in the mare has not been established.

Progesterone is required for the maintenance of pregnancy and is produced by the CL, placenta and adrenal gland. The relative importance of these sources depends on the species and the stage of gestation. In the mare and ewe, progesterone is produced in the latter half of pregnancy by the placenta, while in the cow, sow and goat the CL persists

throughout pregnancy and produces progesterone. Progesterone is permissive to the actions of interferons, growth factors and cytokines responsible for the endometrium to support early embryonic development, implantation and formation of the placenta. Progestogens alter the ionic permeability of the muscles of the myometrium to decrease the excitability of the cells and promote quiescence and non-contractility of the myometrium to avoid abortion. Interferon-stimulated genes in the uterus are involved in nutrient transport, cellular remodelling, proliferation and migration, and protection of the embryo from the maternal immune cells. Supplementing with exogenous progesterone may be useful in maintaining pregnancy.

The embryonic loss rate, which is the difference between the fertilization rate and birth rate, is between 30% and 40% in cattle. Embryonic loss is also the major factor affecting litter size in pigs. The highest rate of embryo loss occurs during the time of maternal recognition of pregnancy. A single treatment with $PGF_{2\alpha}$ between 18 and 28 days postpartum can improve uterine involution and may reduce the number of services to conception. The fertility and pregnancy rate from oestrus induced by $PGF_{2\alpha}$ is about 10% higher than naturally occurring oestrus, possibly due to improved quality of the ova with the shorter period of luteolysis.

Treatment with GnRH once between 11 and 13 days after insemination may delay luteal regression or cause ovulation of the dominant follicle in the next wave and form an accessory CL. This will increase progesterone production and improve the rate of implantation of the embryo and maternal recognition of pregnancy. Treatment with hCG on day 15 improves pregnancy in heat-stressed cows but not in normal cows. Direct treatment with progestogens after insemination can also increase the secretory function of the uterine endometrium and improve the implantation of the embryo.

Pregnancy rates can be increased in mares by delaying the postpartum oestrus. This is accomplished using progestogen treatment either alone or in combination with oestrogen, starting immediately after foaling. Administration of $PGF_{2\alpha}$ can also be used to induce the next oestrus after foal heat. Early embryonic mortality in sheep is increased with very poor body condition, high temperatures in the first week after mating and severe stress. For more information on recognition and maintenance of pregnancy, see the review by Raheem, (2017).

Induction of abortion/parturition

Parturition is the process of delivering a fetus and placenta from a pregnant uterus. It is triggered by the fetus, which releases corticosteroids due to activation of the fetal hypothalamic–pituitary–adrenal axis as the fetus matures (Fig. 5.15). Reducing the production of cortisol by using CRH receptor antagonists (see Section 6.3) can delay parturition. The production of CRH by the fetus and placenta increases during the last week of pregnancy, to increase cortisol production by the fetus in goats, cattle and pigs, and less so in the horse. CRH may be the clock that determines the onset of parturition.

Progesterone production by the CL decreases, and increased levels of corticosteroids stimulate the production of oestrogens by the placenta. Oestrogens stimulate the production of $PGF_{2\alpha}$ by the uterus and increase the number of oxytocin receptors in the uterus. Oxytocin is released by the posterior pituitary in response to the placental membranes entering the birth canal. Oxytocin increases the production of $PGF_{2\alpha}$, and both hormones increase contractions in the uterus. In CL-dependent species, prostaglandin causes regression of the CL and removes the source of progesterone. Treatment with corticosteroids can thus induce parturition if a functional placenta exists. Prostaglandin treatment is needed to induce parturition when there is a non-functional placenta, as in the case of fetal mummification.

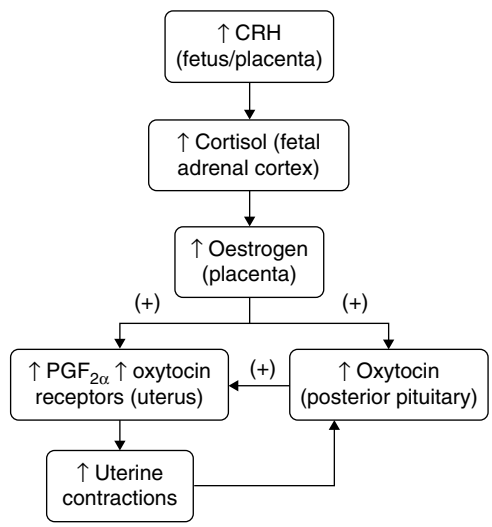


Fig. 5.15. Induction of parturition.

Behavioural changes can indicate that parturition is near. This includes restlessness and increased walking and searching, increased transition from standing to lying, increased tail raising and decreased feeding behaviour and rumination. Cattle and sheep may try to isolate from the herd to find a safe place to give birth and to hide the newborn. Sows exhibit nest-building behaviour about 24 h before parturition. Preventing nest building results in decreased oxytocin and increased cortisol levels, which delays farrowing, so adequate space and nesting materials should be provided.

A wide variety of sensors are available that can measure prepartum behaviours and thus predict the time of parturition. This includes sensors for activity and leg position, eating and rumination, and body temperature. Birthing can be confirmed using intravaginal-temperature and light-sensitive sensors that are GPS enabled and expelled during birthing. These sensors are particularly useful in extensive grazing situations and can be used to locate the newborn. For more information, see the review by Crociati *et al*. (2022).

Abortion is induced in animals entering feedlots, after inappropriate matings and due to pathological pregnancy. Up to 5 months of pregnancy, abortion is induced in cattle using $PGF_{2\alpha}$, while combinations of $PGF_{2\alpha}$ and corticosteroids are used late in gestation. Calves born more than 3 weeks premature have poor viability.

Feeding inhibitors of prostaglandin synthase, such as aspirin or indomethacin, can reduce the levels of prostaglandins. This approach may be useful for inhibiting oestrus or maintaining pregnancy. In postpartum ewes, indomethacin treatment increased LH pulse frequency and amplitude and resulted in an early return to oestrus.

Oxytocin is also used to cause more intense uterine contractions during parturition, for the expulsion of a retained placenta and the acceleration of uterine involution. The cervix must be appropriately dilated before using oxytocin to induce contractions. Oxytocin is also used to induce contraction of the myoepithelium cells in the mammary gland and cause milk let-down.

Postpartum interval

There is normally a period of 25–45 days of anoestrus in dairy cattle and 30–130 days in beef cattle before the first oestrus occurs postpartum. Ovarian cycling can be limited by inadequate LH pulse frequency, so follicles do not mature properly. There can also be a decrease in the normal preovulatory LH surge from positive feedback by oestradiol, due to reduced function of the hypothalamic–pituitary axis. The first ovulation postpartum often results in silent oestrus and a CL that regresses early and results in a short cycle of 8–10 days. This is due to early production of $PGF_{2\alpha}$ by the uterus and points out the need for progesterone priming of the follicles for normal CL function after ovulation. Progesterone priming of the brain is also necessary for normal oestrous behaviour that is induced by oestradiol.

In order to shorten the period of anoestrus, the frequency of LH pulses has to be increased so that the follicles will reach the final stages of maturity. The presence of continuous low levels of progesterone, which do not affect pulsatile LH release but prevent the LH surge, results in large persistent follicles containing ageing oocytes. Other factors, such as high blood cortisol levels, may also block the LH surge. Persistent large follicles can form ovarian cysts, which can be treated by injection of GnRH. Luteal cysts that produce high levels of progesterone can also form and can be treated by injection with $PGF_{2\alpha}$. In addition to limitations on ovulation, the uterus of the postpartum cow requires 30–40 days to complete involution.

The duration of postpartum anoestrus is affected by nutrition, suckling, seasonal factors and the presence of males. In high-yielding Holsteins, ovulation can be delayed by severe negative energy balance, dystocia, retained placental membranes and uterine infections. Some animals, particularly seasonal breeders, are very non-responsive to treatments designed to induce the ovarian cycle and are said to be in 'deep anoestrus'.

Suckling more than two to three times per day, or increased frequency of milking, increases levels of endogenous opioid peptides in the brain, which increases the sensitivity of the hypothalamus to negative feedback from oestrogen. This decreases the production of GnRH by the hypothalamus and the release of LH by the pituitary. The decrease in LH prevents the final maturation of the follicle to the pre-ovulatory stage, with ovarian follicles undergoing repeated waves of atresia prior to first ovulation, and increases the number of days to first oestrus and ovulation. However, suckling also improves uterine involution and increases the conception rate. Sows need to nurse for about 10 days or they will develop follicular cysts and persistent

oestrus and will have poor uterine involution. As lactation progresses, the frequency of LH pulses increases and the maturation of follicles resumes. Weaning the piglets so that the inhibition of LH release from suckling is eliminated, plus exposure to a boar, will stimulate follicular growth and ovulation in sows.

CYSTIC OVARIAN DISEASE About 30% of postpartum dairy cows develop ovarian cysts, which are structures greater than 2.5 cm in diameter that last more than 10 days and are follicular or luteal in origin. Follicular cysts are more common than luteal cysts and are associated with a high level of production, reduced nutrient status, uterine infections, age, stress and season, with a higher incidence in the autumn and winter. They are caused by inadequate LH response to oestradiol and failure of ovulation and are associated with anoestrus postpartum. Stress-induced release of ACTH and corticosteroids can also reduce LH release and cause cyst formation.

High levels of progesterone in the blood or milk can indicate the presence of luteal cysts. Follicular cysts can be treated with hCG or GnRH to induce luteinization of follicular cells and luteal cysts can be treated with $PGF_{2\alpha}$ to cause luteolysis, but fertility of treated cattle is still compromised.

EFFECTS OF NUTRITION The nutrient (energy) balance of the animal is the difference between the availability of nutrients from body reserves and feed intake and the nutrient requirements of the animal for lactation, metabolism, growth and activity. A reduced energy balance can delay puberty and increase the period of postpartum anoestrus. This may be signalled by low levels of blood glucose, which increases the negative feedback by oestradiol on GnRH release by the hypothalamus and decreases the level of LH. The level of IGF-1 is also decreased during negative energy balance, which, together with decreased LH, contributes to decreased follicular growth and maturation. Poor nutrition results in a reduced size of follicles and increased turnover of the dominant follicle. Oestrous cycles in mature cycling pigs can be disrupted in severe and prolonged feed restriction, while less severe conditions will delay puberty, increase the period of postpartum anoestrus and reduce ovulation and embryo survival rates.

Undernutrition results in a decrease in plasma levels of insulin, IGF-1 and leptin and an increase in growth hormone. IGF-1 acts with the gonadotrophins to stimulate follicular development and a low level of IGF-1 in follicular fluid is associated with a low ovulation rate. Insulin stimulates nutrient uptake and prevents apoptosis in granulosa cells, reduces atresia of follicles and increases ovulation rate. Leptin (see Section 3.5) may act to signal the reproductive system that sufficient body fat is present to support a pregnancy. Sufficient levels of leptin are necessary for the normal onset of puberty and cyclic ovarian function. Leptin treatment has been shown to increase sexual development and levels of LH and FSH in male and female rodents. Levels of leptin are correlated with size of the fetus and appropriate levels of leptin are required for the maintenance of pregnancy.

Dairy cattle are normally in a period of negative energy balance during the postpartum transition period, when maximum milk production is reached before maximum feed intake has occurred. The time to first ovulation postpartum is a function of the number of days the cow is in the lowest energy balance; this averages about 33 days in Holsteins in the USA. There is also a period of negative energy balance in beef cattle, even though milk production is much lower than in dairy cattle. Improved feed intake or body condition score will stimulate puberty in young animals and decrease the period of anoestrus in older animals.

Correcting the negative energy balance and improving body condition score will reduce the period of anoestrus in transition dairy cows. These factors contribute to an increase in conception rates after 60 days postpartum. Follicular growth is decreased in lactating cows compared with dry cows. Treatment with bST stimulates the growth of smaller follicles, while feeding the calcium salts of long-chain fatty acids stimulates growth of the largest follicles. Feeding high-energy diets and a high-quality protein also shortens the time to first ovulation in transitional mares that have a low amount of body fat and low body condition score. For more information on the effects of nutrition on cycling in beef heifers and dairy cattle, see Crowe *et al*. (2014); and for pigs, see Tokach *et al*. (2019).

EFFECTS OF STRESS Seasonal periods of heat stress above 32°C increase the length of the oestrous cycle and can reduce plasma levels of oestradiol before oestrus, and thereby decrease the intensity and duration of oestrus. This lowers fertility and oestrus detection rates. Injection of GnRH at oestrus has been suggested as a method to improve the LH

surge and ovulation rate. Progesterone production by the CL is also decreased during heat stress.

Elevated body temperature also increases embryonic mortality. Heat stress decreases feed intake, growth rate and milk production. It can also lower libido and sperm quality in males, resulting in lower fertility. More subtle changes in temperature may also play a role in the timing of the circannual reproductive rhythm of seasonal breeders. Bulls also show seasonal effects on fertility, with improved sperm production during long days and decreased sperm production during high temperatures. Other forms of generalized stress (see Section 6.3) can also contribute to the intensity of seasonal infertility.

Puberty and seasonality

Key concepts

- During puberty, LH pulses increase due to decreased negative feedback from oestradiol.
- Exposure to a male increases the pulsatile release of LH and decreases the age at which puberty is reached in cattle, sheep, goats and pigs.
- Seasonal effects on fertility are due to differences in photoperiod and temperature, with animals being either short-day or long-day breeders.
- Seasonal differences in photoperiod are detected by the pineal gland, which produces melatonin during periods of darkness.
- Kisspeptin, glutamate, NO and neuropeptide Y stimulate the release of GnRH from neurons in the ARC during the breeding season, while dopamine and opioids are inhibitory.
- Regular oestrous and ovulation cycles can be induced outside the breeding season using altered lighting schedules and treatment with melatonin, prostagens, GnRH, dopamine antagonists or $PGF_{2\alpha}$.

Inducing puberty

An animal reaches the puberty stage of sexual maturation when it can display complete sexual behaviour and produce and release gametes. During the onset of puberty, both the amplitude and frequency of LH pulses by the pituitary increase because the hypothalamic–pituitary axis becomes less responsive to negative feedback from oestradiol. Regular oestrous cycles then begin in females with the maturation of the hypothalamic–pituitary axis.

The onset of puberty is influenced primarily by reaching a target age and weight, although the age at puberty varies with the breed, nutritional status and environmental effects, such as photoperiod and temperature. Puberty occurs at 6–7 months of age in sheep, goats and pigs and at around 12 months of age in cattle. A reduced energy balance can delay puberty by decreasing production of GnRH and gonadotrophins. It is thought that leptin normally acts as a signal of energy sufficiency to stimulate the activity of kisspeptin neurons, which in turn activate the GnRH neurons and induce puberty. Other neuropeptides that regulate feed intake (see Section 3.10) may also be involved.

Exposure to a male increases the pulsatile release of LH and decreases the age at which puberty is reached in cattle, sheep, goats and pigs. The presence of a male also shortens the period of anoestrus by acting on the hypothalamus to reduce the negative feedback of oestrogen on LH release. The 'ram effect' is used to induce ovulation in ewes. Male sheep and goats produce pheromones in sebaceous glands, which are released on the hair. Male pigs release sex pheromones in the saliva that induce puberty and cause oestrous females to stand for mating. Further details are given in Section 6.2. In addition to male pheromones, visual, auditory and tactile cues are less important components of the male effect on stimulating female reproduction.

Growth hormone plays an important role in sexual maturation and may accelerate puberty by activating the LH pulse generator or potentiating the action of androgens.

Seasonal effects on reproduction

Seasonal breeding is a common adaptive strategy among mammals, due to varying lengths of gestation, allowing for the birth of young when food is abundant and their survival and growth is more likely. There are seasonal effects on reproductive efficiency in cattle but cattle are not considered to be true seasonal breeders. Season plays a role in the induction of puberty in cattle, with heifers born in the autumn reaching puberty before those born in the spring. Animals born in the autumn are from 6 to 12 months of age when spring arrives, and the increased photoperiod during the spring stimulates the hypothalamic–pituitary–ovarian axis. Seasonal differences in photoperiod can affect the period of postpartum anoestrus by 10–35 days. Cows calving in the period of lengthening photoperiod from late

spring to early autumn have a shorter period of anoestrus than animals that calve in the winter, when the photoperiod is decreasing.

The domestic pig is capable of producing piglets throughout the year, although there is reduced fertility in the late summer and early autumn. During this time, the seasonal-breeding European wild boar is in seasonal anoestrus. Seasonal infertility results in a reduced farrowing rate, delayed puberty in gilts and a prolonged weaning to oestrus interval.

Seasonal effects on fertility are due mainly to differences in photoperiod and temperature. There is generally little seasonal effect on reproduction in breeds that are native to the subtropics and tropics, which are not exposed to dramatic seasonal changes in photoperiod or temperature.

True seasonal breeders include sheep, goats and horses, as well as water buffalo, which are important in Asia, and caribou and reindeer, which are raised in northern regions. Sheep, goats and deer are short-day breeders, with increased reproductive activity during the shorter days in the autumn, while horses and most poultry are long-day breeders, with increased reproductive activity in the longer days of the summer. During the anoestrous period, oestradiol strongly inhibits the secretion of GnRH by negative feedback, which limits LH release from the pituitary. The development of follicles can continue in some species during the period of anoestrus but the levels of LH are insufficient for the final stages of follicular development. In other seasonal breeders, follicular development ceases during the anoestrous period. Testis size and sperm production are also reduced in rams during the non-breeding season. Season affects the time to reach puberty in sheep, with ewe lambs born in the autumn not showing oestrus until after the summer anoestrus.

During the breeding season, the strong negative feedback of oestradiol on GnRH secretion is reduced. The change in photoperiod increases LH pulse frequency, and FSH levels increase to stimulate the production and maturation of large, active follicles. Eventually, a dominant follicle develops and ovulation occurs. The first oestrus that occurs in mares during the spring transition period after the winter anoestrus is typically erratic and long in duration and it is difficult to determine the time of ovulation.

Regulation of LH production

Seasonal differences in photoperiod are detected by the pineal gland, which receives impulses from light sensory neurons in the retina of the eye by way of excitatory neurons of the master clock in the suprachiasmatic nuclei (SCN) of the hypothalamus, which generates a 24 h rhythm (see Section 1.4). The pineal gland releases melatonin (*N*-acetyl-5-methoxytryptamine, Fig. 5.16) during periods of darkness, but not during light periods, and the pattern of melatonin release regulates the pulsatile release of GnRH from the hypothalamus and in turn the production of pituitary gonadotropin and gonadal steroids. In this way, the pattern of melatonin release regulates seasonal patterns of reproduction, with increased melatonin secretion in short days of winter and decreased melatonin secretion in long days of summer. Melatonin inhibits GnRH release in long-day breeders, such as the mare, so the decreased levels of melatonin due to long days increases GnRH release from the hypothalamus. Conversely, increased melatonin due to short days stimulates GnRH release in short-day breeders such as sheep and goats.

Melatonin is synthesized in the pineal gland from serotonin, which is acetylated by serotonin *N*-acetyltransferase (NAT) and then methylated by hydroxyindole–*O*-methyltransferase to yield melatonin (Fig. 5.16). NAT is the rate-limiting enzyme in the biosynthesis of melatonin and its activity is low during daylight and peaks during the dark phase.

Melatonin acts via its G protein-coupled receptor (MTNR1A) in the anterior pituitary to decrease production of thyroid-stimulating hormone (TSH). During long days, decreased levels of melatonin elevate TSH levels, which increases the hypothalamic expression of type II iodothyronine deiodinase (DIO2), which converts T_4 to the active T_3, and decreases the expression of DIO3, which metabolizes T_4 and T_3 to inactive reverse T_3 (rT_3) and 3,5 diiodothyronine (T_2), respectively. During short days, the increased levels of melatonin decrease the activity of DIO2 and increases the activity of DIO3, which results in decreased levels of T_3 in the mediobasal hypothalamus. The T_3 concentration within the mediobasal hypothalamus is about tenfold higher during long days than short days, even though plasma concentrations are similar in both photoperiods. T_3 in the hypothalamus activates genes encoding the neuropeptides GnIH and kisspeptin (KISS1), which regulate the secretion of GnRH. T_3 also alters the morphology of the GnRH neurons to affect the secretion of GnRH from the median eminence. T_3 improves contact of the GnRH nerve terminals with the pericapillary

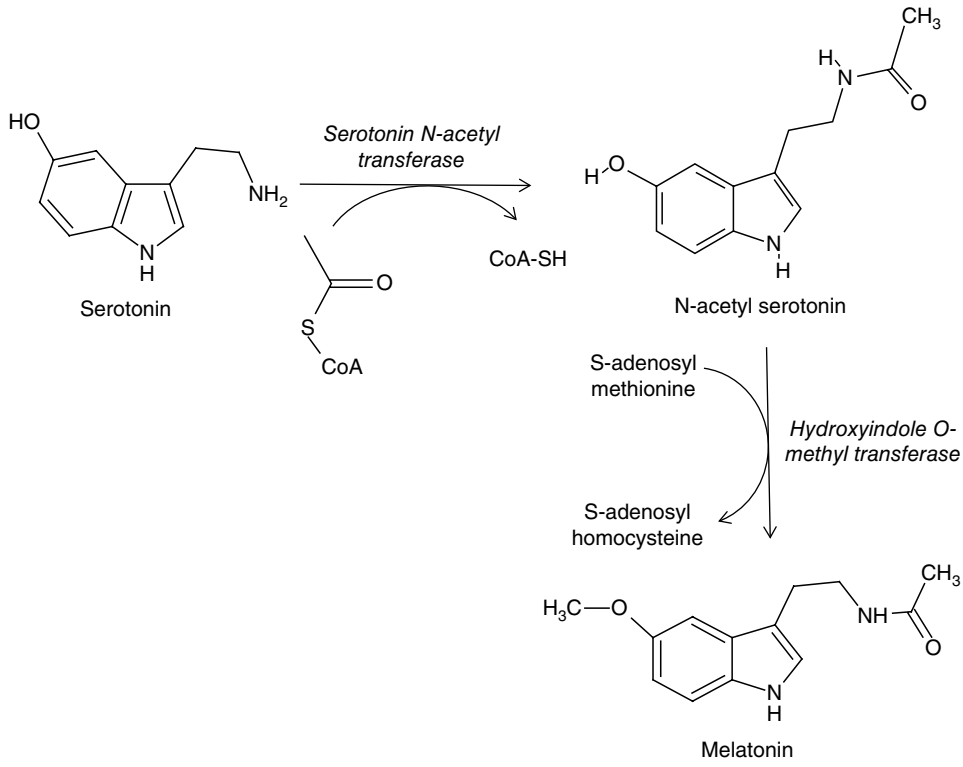


Fig. 5.16. Biosynthesis of melatonin.

For interest

Polymorphisms in the melatonin receptor gene *MTNR1A* are associated with reproductive seasonality and litter size in sheep (Pulinas *et al*., 2022).

space to allow GnRH to reach the portal capillary system. During short days when melatonin levels are high and T_3 levels are low, many GnRH nerve terminals are encased by the end-feet of glial processes and do not contact the basal lamina, so GnRH cannot reach the portal system. Melatonin does not act directly on the GnRH neurons but acts indirectly through other neurons that finally synapse on the GnRH neurons.

Kisspeptin neurons in the ARC are involved in the seasonal regulation of GnRH neurons as well as in the induction of puberty, while the preovulatory stimulation of GnRH neurons that drives ovulation occurs in the POA (see above). The activity of kisspeptin neurons and their input to GnRH neurons in the ARC decrease in the non-breeding season and increase during the breeding season, in concert with the activity of GnRH neurons. Reproductive function in sheep can also be restored during the non-breeding season by treatment with kisspeptin. GnRH neurons do not have oestrogen receptor α and the seasonal response of GnRH release to oestrogen feedback is due to changing responsiveness of the kisspeptin neurons to oestrogen. Kisspeptin neurons do not express the melatonin receptor, so the effects of photoperiod on these neurons may involve suppression by dopamine during the non-breeding season. For more information on seasonality of reproduction and the role of kisspeptin in seasonality, see the reviews by Beltran-Frutos *et al*. (2022), Reiter *et al*. (2018) and Shinomiya *et al*. (2014).

Dopamine is involved in the negative feedback of oestrogen on neurons that produce GnRH (Fig. 5.17) and injection of dopamine antagonists (pimozide, domperidone, perphenazine, sulpiride) increases LH secretion in seasonally anoestrous ewes and mares. Opioids, such as β-endorphin, are also involved in the reduced gonadotrophin secretion in seasonally

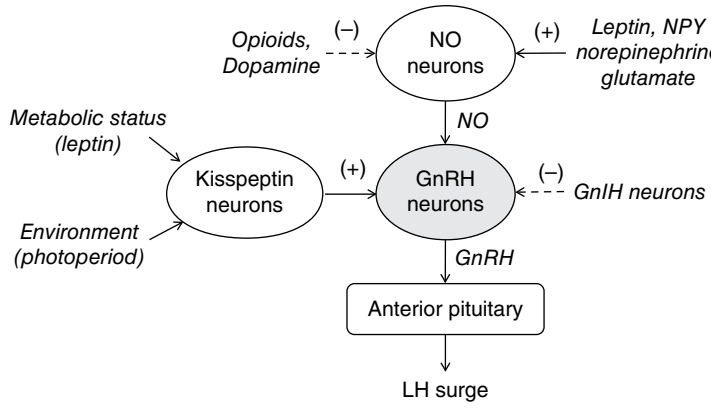


Fig. 5.17. Control of LH release.

anoestrous mares and administration of the opioid antagonist naloxone advances the first ovulation of the year. Opioids are also thought to reduce the production of GnRH in cycling animals following ovulation, while glutamate, NO, carbon monoxide and neuropeptide Y stimulate neurons that produce GnRH.

NO is a gaseous chemical messenger that acts locally in tissues and is involved in vascular relaxation, activation of the immune system, platelet function and as a neurotransmitter. NO is synthesized from arginine by the calcium-sensitive enzyme nitric oxide synthase (NOS). NOS exists in a few different tissue-specific isoforms, including a neuronal nNOS, an inducible iNOS in immune cells and endothelial eNOS. NO is involved in the LH surge mechanism and also acts in the ovary to regulate steroidogenesis, ovulation and luteolysis. NO is produced by nNOS in specific neurons and diffuses to adjacent GnRH neurons. In the GnRH neurons, NO activates soluble guanylate cyclase to increase levels of cGMP and cylco-oxygenase to increase synthesis of prostaglandins ($PGE_{2\alpha}$), which leads to increased release of GnRH. Leptin acts via increased NO in the hypothalamus and pituitary to increase release of LH. Leptin is also necessary for the production of kisspeptin, which stimulates GnRH production. Glutamate and norepinephrine neurons also stimulate NO production. In the ovary, the activity of NOS increases during the preovulatory LH surge, stimulating prostaglandin production and the inflammatory process at ovulation. For more information, see the review by Chachlaki and Prevot (2020).

Advancing cyclicity in seasonal breeders

In seasonal breeders, such as sheep and horses, regular oestrous and ovulation cycles can be induced at the beginning of the breeding season. The official yearling date of foals for each breeding season is set as 1 January of the year following their birth by many breeding associations, so it is advantageous to breed mares early in the year to have an age advantage over foals born later in the season. Increasing the photoperiod using artificial lighting is used to induce oestrus in mares. About 14–16 h of light exposure for 8–10 weeks is used, starting at the beginning of December to induce oestrus by the middle of February. Supplemental lighting is given at the beginning and end of the natural daylight. A 1–2 h light period 9.5 h after the beginning of the dark period has also been suggested as a method to induce oestrus, suggesting that this light-sensitive period plays an important role in sensing circannual changes in photoperiod (see also Section 4.2: 'Regulation of follicular development and egg production'). In sheep and goats, which are short-day breeders, the photosensitive phase occurs 14–16 h after dawn. Sheep and goat AI centres maintain high rates of semen production throughout the year by alternating between 1 month of long days and 1 month of short days. On goat farms, males and females are exposed to the same alternating lighting schedules to maintain reproductive prolificacy.

Melatonin is involved in the regulation of circadian rhythms, including the sleep–wake cycle in humans. In the absence of external signals, these

rhythms would be free running, but they are normally synchronized to the endogenous melatonin and photoperiod signals. Administration of exogenous melatonin or inhibition of melatonin synthesis by exposure to bright light can be used to entrain these physiological rhythms to new signals. This can be used to treat sleep–wake disorders in workers on different shifts, due to changes in time zone (jet lag), in blind individuals who are unable to perceive light and in elderly people with insufficient melatonin production. In amphibians, fish and some reptiles, melatonin causes aggregation of melanin granules in melanocytes, resulting in blanching of the skin. Seasonal anoestrus can be reduced using melatonin for short-day breeders or increased lighting through artificial lighting programmes for long-day breeders.

A decrease in thyroid hormones occurs during seasonal anoestrus in ewes but thyroid hormones do not seem to be involved in seasonal anoestrus in mares. Melatonin also inhibits prolactin release in ewes, and administration of exogenous prolactin decreases the time to first ovulation in seasonally anoestrous mares.

In ewes, seasonal anoestrus involves an increased sensitivity of the hypothalamus to negative feedback of oestrogen, which is mediated by melatonin (see above). Exogenous melatonin can be given to ewes by injection or feeding to mimic the effects of short days and stimulate gonadal activity. Long treatment periods are needed and a commercial implant of melatonin (Regulin®, Schering Pty Ltd) is available. Boluses of slow-release melatonin that remain in the rumen for extended periods of time can also be used.

Mares can be treated with progestogens (injection of progesterone in oil or feeding Altrenogest® for 10–15 days) to induce oestrus. Progestogen treatment suppresses LH release and may allow LH stores to increase in anoestrous mares, so that sufficient LH will be released to induce ovulation after progestogen treatment has ended. The effects of progestogen treatment are additive to the effects of artificial lighting in inducing oestrus. Treatment with progestogens is more effective in mares during the latter part of the transition period, when larger follicles are present, compared with the early transition period, when there are only small follicles. Oestradiol treatment can be used together with the progestogen treatment to further reduce the variation in follicular development and ovulation. Treatment with dopamine antagonists (domperidone, 1.1 mg kg^{-1} orally or sulpiride 0.5 mg kg^{-1} intramuscularly) (see Fig. 5.17) reduces the negative feedback on LH release and results in earlier time of ovulation in mares, especially when combined with light treatment. The period of treatment is longer for mares that are in deep anoestrus compared with mares in transition.

There is an extended period of oestrus in mares, with ovulation occurring from 1 to 10 days after oestrus begins. Oestrus can be induced using $PGF_{2\alpha}$, as described above for cattle, but the time of ovulation is still variable. The most effective method is treatment with progestogen/oestradiol, followed by $PGF_{2\alpha}$ to remove any persistent CL. Ovulation of large preovulatory follicles (35 mm in diameter) is then induced with hCG. The GnRH analogue deslorelin (Table 5.1) can also be used as an implant to shorten the time to ovulation in mares. For more information, see Williams *et al.* (2012) and Sen and Hoffmann (2020).

Immunological control of reproduction

Key concepts

- Immunizing against inhibin or gonadal steroids can increase ovulation rates.
- Immunization against GnRH, FSH or LH produces a form of non-surgical castration.
- Vaccination against purified ZP glycoproteins or sperm-specific proteins produces antibodies that inhibit sperm function, binding and penetration of the ZP and results in immunocontraception.

Immunization strategies have been developed to increase fertility. Immunization against inhibin increases ovulation rates in sheep and horses by increasing FSH levels. Immunization against oestradiol or androstenedione can also be used in sheep to increase the ovulation rate by removing the negative feedback of gonadal steroids on the hypothalamus and pituitary. A commercial product (Fecundin®, Glaxo Animal Health) uses a dextran adjuvant that can be given as a single injection and produces a moderate antibody response. Synchronization of oestrus is not necessary, but otherwise fertilization rates and embryo survival rates can be decreased. In anoestrous ewes, follicular development is stimulated and multiple ovulations occur when the ewes are exposed to a ram.

There are multiple points in the reproductive process that may be targeted for immunological intervention to reduce fertility (Fig. 5.18). Neutralization of

GnRH, LH or FSH will interfere with the production of gametes (sperm and oocytes) and thereby inhibit fertility. Immunizing against unique antigens in the sperm and zona pellucida (ZP) of the egg can block fertilization. Immunization against chorionic gonadotrophin (CG) that is produced by the embryo to maintain progesterone production by the CL can interfere in implantation of the blastocyst.

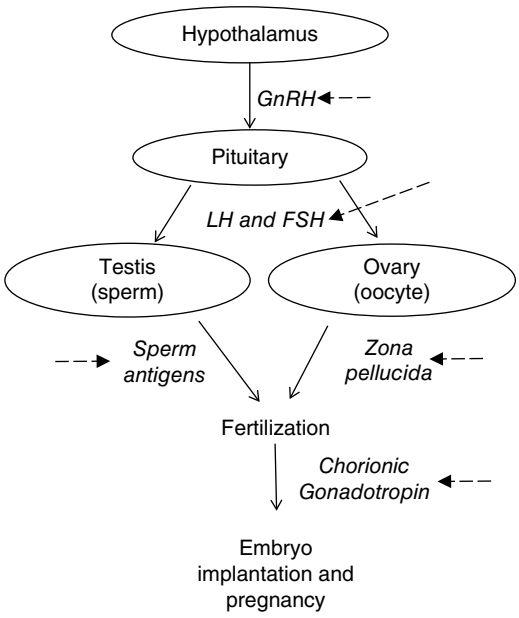


Fig. 5.18. Targets for immunological intervention to reduce fertility.

Immunization against GnRH produces a form of non-surgical castration by neutralizing the effects of GnRH and inhibiting the production of gonadotrophins and gonadal steroids (Fig. 5.19). This effect occurs in both sexes and, depending on the vaccine, active vaccination can last from 1 to 2 years; it is temporary if passive immunization is used. GnRH is available for antibody binding while it is in the hypothalamic–pituitary portal blood vessels on its way from the site of production in the hypothalamus to the anterior pituitary gland. To generate antibodies, GnRH or a GnRH analogue is coupled to a large molecule, such as albumin, keyhole limpet haemocyanin (KLH), or diphtheria or tetanus toxoid (see Section 2.1). Adjuvants other than Freund's complete adjuvant are used commercially, since Freund's causes severe local inflammation at the injection site.

Immunization against GnRH in males reduces testicular size and function and can be used to control aggression, reduce the incidence of male-associated odours and reduce fertility. A commercially available vaccine (Vaxstrate®), based on a carboxyl–GnRH analogue coupled to ovalbumin that was designed for beef heifers, is also effective in male goats and sheep. Bull calves immunized against GnRH had less carcass fat, increased loin eye area and improved weight gain and feed conversion efficiency compared with steers. This is probably due to the increased exposure to testicular steroids in immunized calves compared with castrates. Immunized lambs produced carcasses that were similar to castrates but had similar back fat to intact control lambs. Immunized bucks and boars

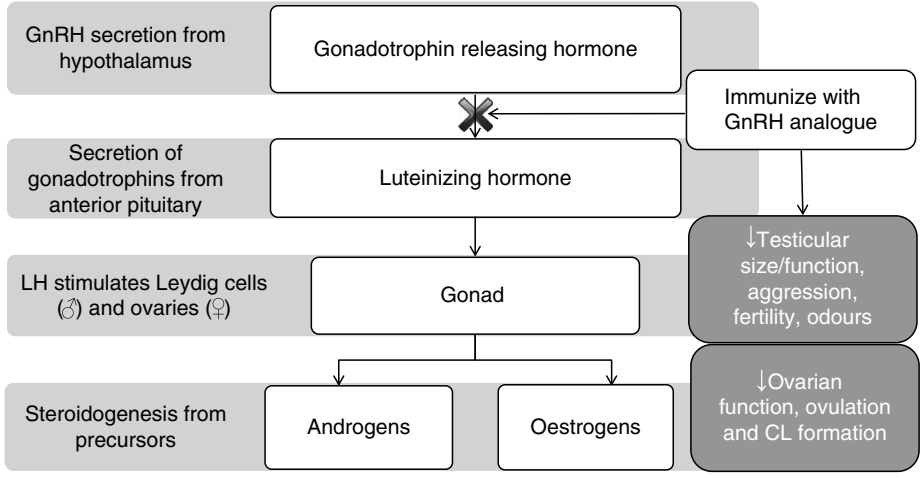


Fig. 5.19. Effects of immunizing against GnRH.

had decreased odour score but similar carcass weights as controls. A tandem repeat of GnRH linked to KLH produced a more consistent immune response in boars and a commercial vaccine (Improvac®) is available to prevent boar taint. The effects of immunocastration on carcass composition depend on the vaccination protocol and age of the animal. Immunization protocols have been developed that consistently reduce boar taint and do not adversely affect the improved feed efficiency of intact boars or the lean content of boar carcasses compared with castrates (see Section 3.3). The vaccine has also been used in female pigs that are grown to heavier carcass weights. This directs dietary nutrients to growth and suppresses ovarian function to reduce sexual behaviour and reduce unwanted pregnancies in gilts intended for slaughter.

An alternative approach for reversible antifertility treatment is to deliver a GnRH agonist (deslorelin) in a slow-release implant. This decreases GnRH production by negative feedback to decrease LH, testosterone, testicular volume and semen output.

Follicle-stimulating hormone (FSH) plays an essential role in the initiation and maintenance of spermatogenesis, with the FSH-β subunit responsible for binding the FSH receptor. Immunization of male bonnet monkeys using ovine FSH resulted in testicular dysfunction, oligozoospermia and subsequent infertility that was not associated with any change in testosterone levels.

An acute rise of LH triggers ovulation and development of the CL in females and LH stimulates testosterone production by Leydig cells in males. Immunization of adult female sheep with LH blocked pregnancy during two breeding seasons. Immunization against LH would also affect the production of sex steroids.

The fertility of females can be reduced by vaccination with a key target protein that permanently affects the reproductive integrity of the ovary. The zona pellucida (ZP) is a glycoprotein matrix surrounding the oocyte that has receptor sites for sperm binding. If the ZP is masked or altered, fertilization will not occur. Vaccination with purified ZP glycoproteins or synthetic peptides representing specific B-cell epitopes produces antibodies that inhibit sperm binding and penetration of the ZP and results in immunocontraception. Because the ZP glycoproteins are highly conserved across several phyla, the vaccine is effective in multiple species and can be used for reproductive management of wildlife. Antibody titres normally fall over a period of time after vaccination and this can lead to an eventual increase in fertility in mature animals that have been immunized against important reproductive proteins such as ZP glycoproteins. This also occurs in animals that are immunized against GnRH, but the return to fertility is more rapid than with ZP vaccines. Immunosterilization will result if the immune reaction causes irreversible damage, such as the atrophy of follicles or the destruction of oocyte/granulosa cell complexes.

Contraceptive vaccines that affect sperm function should target protein antigens that are sperm specific and have a fertility-associated function. These proteins can be involved in sperm motility, ability to capacitate, the acrosome reaction, penetrating ZP, and finally fusion with the egg. The proteins should also be immunogenic and accessible to antibody binding.

Chorionic gonadotrophin (CG) is formed by the trophoblast after fertilization and acts on the CL to stimulate progesterone production, which prepares the uterine endometrium for implantation of the embryo. Levels of hCG increase during pregnancy and this is the basis of the pregnancy test in humans. A similar hormone, equine chorionic gonadotrophin (eCG), also known as pregnant mare's serum gonadotrophin (PMSG), is involved in pregnancy recognition and embryo implantation in the mare. eCG has only LH-like activity in horses, but in other species it has both FSH and LH activity. Both hCG and eCG are used to induce oestrus in sheep, goats, cattle and pigs. Vaccines have been developed against hCG to prevent pregnancy using a variety of different carriers, including diphtheria toxin (DT), tetanus toxin (TT) and enterotoxin subunit B (LTB). No side effects were observed during Phase I and Phase II trials, as women showed normal ovulation and normal synthesis of their own sex hormones along with regular menstrual cycles. For more information, see Gupta and Bansal (2010) and Kaur and Prabha (2014).

5.2 Endocrine Manipulations in Aquaculture

Key concepts

- Sex control is used in aquaculture to improve growth, feed efficiency and carcass quality and prevent interbreeding with wild fish stocks.
- Oestrogen is used for feminization of fish, while 17α-methyl testosterone is used for masculinizing fish.

- Exogenous steroid hormones are most effective during the 'labile period' of sexual development, when the gonads are still undifferentiated.
- Indirect methods first masculinize females or feminize males, and then breed them to produce all XX female or all XY male offspring.
- Sterile triploid fish can be produced by preventing extrusion of the second polar body after fertilization, using heat, pressure or anaesthetic chemical treatment.
- Spawning time can be induced by changing photoperiod and treatment with GnRH and dopamine antagonists.
- Growth hormone is the most potent growth promoter in teleost fish. It acts in synergy with androgens and thyroid hormones to increase growth.
- Compensatory growth following feed restriction results in improved lean growth and feed efficiency and decreased fat deposition.
- Long-term stress can reduce growth performance and reproductive success and increase the susceptibility to disease due to elevated levels of corticosteroids.

Fish is an important part of the human diet and fish meal is an important commodity as a component of animal feeds. Aquaculture is increasingly needed, since natural sources of fish are unable to supply the demand for this commodity. Improved reproductive management, growth performance and feed efficiency of aquaculture systems can be achieved by endocrine manipulations.

Control of reproduction

Sex differentiation and maturation

Sex is determined by the presence of sex chromosomes (either XX/XY or ZW/ZZ) in most species of fish of commercial interest. Sex differentiation in fish can occur by direct differentiation of the primordial gonad into either a testis or an ovary. This occurs in 'differentiated' species, such as the medaka, coho salmon, common carp and European sea bass. In 'undifferentiated' species, such as the guppy, hagfish and European eel, the gonad first develops as an ovary-like structure and then forms a testis in some of the fish.

The major genes involved in sex differentiation include anti-Müllerian hormone (*amh, amhy*) and the amh receptor (*amhr2*), doublesex and Mab-3 related transcription factor (*dmrt1*), aromatase (*cyp19a*), factor in the germ line alpha (*figla*), growth differentiation factor 6 (*gsdf*) and SRY-related HMG box (*sox9*). Sex differentiation is also affected by environmental factors including temperature, pH, population density, oxygen concentration, stress and social status.

Oestrogens, specifically oestradiol-17β, are essential for female development. The ovarian thecal layer produces testosterone and other precursor androgens, and the granulosa layer converts testosterone to oestrogen via the enzyme aromatase (CYP19a). Teleost fish have two isoforms of aromatase; one isoform is found primarily in the granulosa cells of the ovary (CYP19a or CYP19a1) and the other neural aromatase (CYP19b or CYP19a2) is localized primarily in the brain and is correlated with seasonal changes in gonadal activity and growth.

Oestrogen is essential for female sex differentiation and the lack of oestrogen allows for male sex differentiation. The incubation temperature of the eggs of reptiles and some fish determines the gonadal sex, and high temperatures tend to produce more males. This has been related to stress and up-regulation of cytochrome P450 11β-hydroxylase (CYP11b), which is involved in the synthesis of cortisol and the major androgen 11-ketotestosterone (11-KT). Down-regulation of aromatase expression by hypermethylation of the aromatase promoter at higher temperature has also been shown. Changes in oestrogen synthesis in response to temperature occur first in the brain and later in the gonads. The transcription factors FOXl2 and SF-1 up-regulate the expression of aromatase in fish and this sexual dimorphism in SF-1 expression is reversed in mammals (see Section 5.1).

Male sex differentiation is linked to the androgens 11-KT, 11β-hydroxyandrostenedione and 11β-hydroxytestosterone, with 11-KT the major circulating androgen responsible for testicular development. The conversion of androstenedione and testosterone into these 11-oxygenated steroids is catalysed by CYP11b, which is also a key enzyme in the synthesis of the stress hormone cortisol. Interstitial cells of the testis, which are analogous to Leydig cells, are the major site of androgen synthesis. In males, the transcription factor DMRT1 is involved in normal testis differentiation and decreases the expression of aromatase and increases the expression of 11β-hydroxylase.

Sex reversal

Altering the steroid-synthesizing capability can disrupt sex determination and exogenous steroids can be used for sex reversal in many fish, amphibians and reptiles. Exposing the young fry to exogenous hormones can readily alter the phenotypic sex of fish. Sex control is used in aquaculture to produce the sex of fish that grows more rapidly; this is female in the salmonids and the cyprinids, while it is male in the cichlids. Sexual maturation in males is inhibited in production systems, in order to reduce problems with poor carcass quality and aggressive and territorial behaviour in sexually mature fish. Both males and females are sterilized to allow larger fish to be reared and to prevent poor feed efficiency, as nutrients are normally diverted into gonadal growth as the fish matures. In sea ranching, rearing sterile fish prevents interbreeding of wild fish stocks by captive fish that may escape.

Sex steroids regulate the differentiation of the gonads in fish. Oestrogens are responsible for ovarian differentiation; treatment of genetic female salmon with an aromatase inhibitor will cause them to develop into functional males. Environmental factors can also affect sexual differentiation by inducing or repressing the activity of certain genes, such as aromatase. Similarly, treatment of genetic female chickens with aromatase inhibitors early in development results in the development of testes rather than ovaries (see Section 4.2). Treatment of genetic male marsupials with oestrogen early in development causes the development of ovaries.

Fish can be directly feminized by treatment of a group of sexually undifferentiated fish with oestrogen but half of these phenotypic females will have a male genotype. There may also be some concern from consumers about the direct use of steroid treatment in fish destined for human consumption.

HORMONAL TREATMENTS FOR SEX REVERSAL Acute administration of hormones to fish can be achieved by injection of the hormone (intramuscularly or into the body cavity) or immersing the fish in water containing the hormone. Feeding hormone-treated food or implantation of silastic capsules, cholesterol pellets or osmotic minipumps has been used for chronic administration of hormones. Addition of hormone to the water is useful for treatment of larval stages, when the hormone can be absorbed via the gills or integument. Rearing density is important with this method. Administration of the hormone in the feed, which avoids disturbing fragile larvae, can be used once external feeding has begun.

Oestrogenic compounds, particularly oestradiol, are used for feminization of fish, while 17α-methyltestosterone is used for masculinizing fish. High doses or long exposure times to the hormone result in sterilization. In Atlantic salmon, doses of > 20 mg kg^{-1} of 17α-methyltestosterone for the first 600 degree-days of feeding results in sterilization. Several endocrine disruptor chemicals have also been shown to affect sexual development in fish (see Section 6.4). Other oestrogenic compounds, such as diethylstiboestrol (DES), are not used, since they are carcinogenic in humans. In the EU, Council Directive 2008/97/EC regulates the use of sex steroids for sex control in finfish aquaculture.

Treatment with oestrogens can sometimes alter male secondary sex characteristics without complete sex reversal. High levels of oestrogen can also reduce survival and growth rate. Salmonids require the lowest exposure (a combination of dose and treatment duration) to steroids for sex reversal, while cyprinids require considerably more exposure. In many species of salmonids, steroids are administered by immersion of the newly hatched fry in water containing the steroid. This probably allows the steroids to accumulate in the yolk sac, where they will continue to affect the developing fish until day 20.

Exogenous steroid hormones are most effective during the 'labile period' of sexual development, when the gonads are still undifferentiated. The timing of this labile period is species specific and somewhat dependent on the hormone used and the dose. For example, the labile period for oestrogen occurs before the labile period for androgens. Treatment times as short as hours have been used. In coho and chinook salmon, one or two 2 h immersions in steroids (400 μg l^{-1}) during the alevin stage at 8–13 days after hatching is sufficient to cause sex reversal. In tilapia, 30–40 ppm of 17α-methyltestosterone is given in the food for 30 days at the swim-up stage to produce all-male fish for grow out. Treatment outside the labile period requires higher doses of hormone or longer periods of treatment to achieve sex reversal. The optimum treatment protocols for sex reversal use the minimum dose of natural hormone during the labile period, so that the shortest exposure time, and consequently the least effect on survival or growth rate, can be used.

Steroids are metabolized and cleared through the liver (see Section 1.2) and this occurs rapidly, so levels of exogenous steroids would be undetectable in a few days after treatment. Treatment of fish with steroids for sex reversal differs from use of steroids in beef cattle for weight gain (see Section 3.2). Cattle are usually treated with synthetic steroids and the treatment is continued for months and ends very near slaughter. In contrast, fish are usually treated with natural steroids, the treatment is short term and ends long before the fish are marketed, even with the direct method for sex reversal. However, it is likely that indirect methods for sex reversal would be more acceptable to consumers than direct methods.

INDIRECT METHODS All-female fish can also be produced in two generations using an indirect feminization procedure (Fig. 5.20). This indirect procedure does not involve direct treatment of the fish used for food consumption. In this procedure, a group of genetic females are first masculinized by treatment with androgens. These 'neomales', which carry only X chromosomes, are identified using sex-specific DNA probes and bred with normal females. The resulting offspring are all female. The stock is maintained by masculinizing a small number of fish to provide more neomales. The untreated fish are grown out and marketed or used as female brooding stock. Thus, the indirect method of producing all-female stock is more time consuming but does not expose the marketable fish to steroids. It is used commercially for the culture of several salmonids, including rainbow trout and chinook salmon, and is applicable to fish species with the XX/XY sex-determination system.

All-male stocks can also be produced by indirect treatment with oestrogens (Fig. 5.21). Sexually undifferentiated fish are first treated with oestrogens and the 'neofemales' (genetic males but phenotypic females) are bred with normal males. One-quarter of these progeny will be YY 'supermales'. These YY males are identified and bred with normal females to produce 100% XY male progeny. YY males have been produced in several species, including medaka, goldfish, rainbow trout and Nile tilapia, but are not viable in other species. In ZW/ZZ sex-determination systems, as in the black molly, oestrogen treatment of undifferentiated fish produces ZZ neofemales. These are bred to normal ZZ males to produce all-male offspring.

Sterile fish can be produced by inducing triploidy. Triploids can be produced indirectly by crossing tetraploids with diploids or directly by preventing the extrusion of the second polar body after fertilization by heat, pressure or anaesthetic chemical treatment. For example, triploids can be induced in Atlantic salmon eggs by exposure to 30°C for 6–10 min at 20–30 min after fertilization. Pressure treatment is more costly but less variable and can be used to produce triploids over a wider period of time than heat treatment. Exposure of eggs to hyperbaric nitrous oxide gas also produces triploids but the treatment must begin immediately after fertilization and is intermediate in effectiveness between heat and pressure treatment. For more information, see Piferrer (2001) and Martinez *et al*. (2014).

Induction of spawning

Most fish spawn seasonally in the wild in response to environmental cues that occur with the seasons. In temperate and subtropical species, changes in water temperature and photoperiod dramatically affect gonadal growth and spawning. This allows

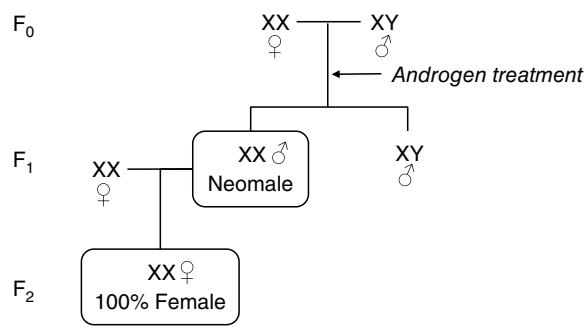


Fig. 5.20. Indirect method for feminization (adapted from Piferrer, 2001).

the progeny to be produced in the spring, when the conditions for their survival are more favourable. In captivity, it is necessary to control spawning time in order to provide marketable fish throughout the year. In the red drum (*Sciaenops ocellatus*), the age to sexual maturity can be reduced by more than half by rearing the fish at high water temperatures. In salmonids, photoperiod is the main environmental cue for gonadal maturation and spawning.

ENDOCRINE CONTROL OF GAMETOGENESIS Gametogenesis (spermatogenesis and vitellogenesis) and the final maturation of gametes (spermiation and oocyte maturation) in fish are regulated by hormones of the hypothalamic–pituitary–gonadal axis (Fig. 5.22). As occurs in other species, GnRH released from the hypothalamus controls the release of LH and FSH by the pituitary. In some fish species, dopamine acts as a GnRH antagonist to prevent the release of gonadotrophins from the pituitary. LH and FSH regulate the production of androgens, oestrogens and progestagens in Leydig cells, while FSH regulates Sertoli cell support of spermatogenesis. Gonadotrophin-inhibitory hormone (GnIH)

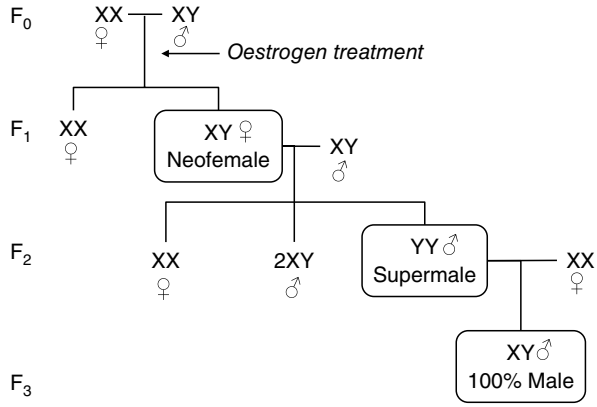


Fig. 5.21. Method to produce all-male stocks (adapted from Piferrer, 2001).

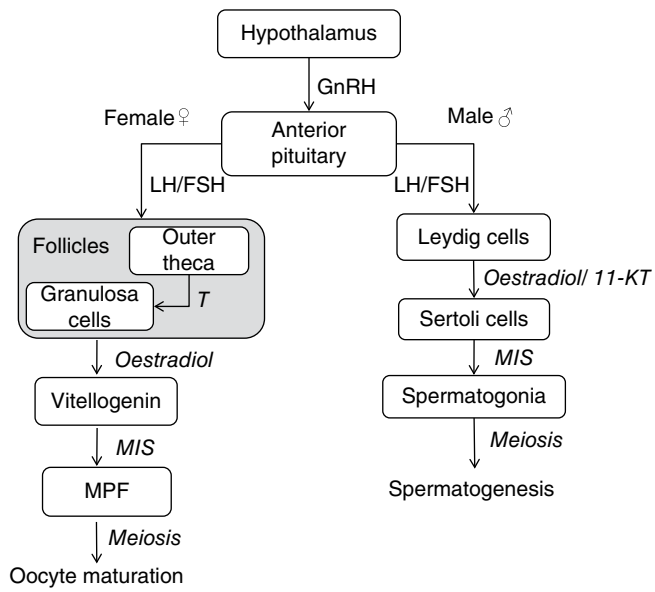


Fig. 5.22. Regulation of gametogenesis in fish. MIS, maturation inducing steroid; MPF, maturation promoting factor; 11-KT, 11-ketotestosterone; T, testosterone.

and kisspeptin exhibit inhibitory and stimulatory effects on GnRH, respectively. In most teleost fish, there are three variants of GnRH (GnRH I–III), while some have only two, which are GnRH II and GnRH III. GnRH III knockout in zebrafish resulted in a male sex-biased population by up-regulating the expression of genes involved in male gonad development, such as *sox9a*, *amh* and *cyp11*.

The maturation-inducing steroid (MIS), which in salmonids is 17α,20β-dihydroxy-4-pregnen-3-one and in some marine species is the trihydroxy derivative 17α,20β,21-trihydroxy-4-pregnen-3-one, is responsible for inducing final maturation of oocytes, and meiosis in spermatogonia. There are two forms of cytochrome P450c17: CYP17a1, which catalyses the 17α-hydroxylase and C17-20 lyase activities required for androgen and oestrogen synthesis; and CYP17a2, which has only 17α-hydroxylase, lacks C17-20 lyase activity and is involved in the synthesis of MIS. Decreased expression of CYP17a1 and aromatase (CYP19a1) and increased expression of CYP17a2 and 20β-hydroxysteroid dehydrogenase (20β-HSD) result in increased synthesis of MIS. For more information, see Hasegawa *et al.* (2022).

In males, the renewal of spermatogonial stem cells by mitosis is regulated by oestradiol acting on Sertoli cells, while the proliferation of spermatogonia to meiosis is promoted by the androgen 11-KT and the growth factors IGF-1 and activin B. Meiosis in spermatogonia is induced by MIS, which also increases at spawning to induce sperm maturation by activating carbonic anhydrase in the spermatozoa. This increases the pH of the seminal plasma, which increases levels of cAMP in the sperm to induce sperm motility and capacitation.

During vitellogenesis in females, oestradiol is produced by the developing follicles to regulate oocyte development and the synthesis of vitellogenin and other yolk proteins by the liver. Gonadotrophins increase steroidogenesis in follicles, with testosterone produced by the outer theca layer being converted to oestradiol by the granulosa cells. At the conclusion of vitellogenesis, oocyte maturation is induced by LH, which stimulates the production of MIS by the follicle cells. MIS inhibits adenylate cyclase to decrease cAMP-dependent protein kinase activity in the oocyte. This activates 'maturation promoting factor' (MPF), which is a complex of an active CDC2 kinase and its regulatory subunit cyclin b, to stimulate the resumption of meiosis and completion of oocyte maturation (see Fig. 5.22).

APPLICATIONS Environmental and hormonal manipulations can be used to manipulate spawning time (Fig. 5.23). The annual cycle of temperature and photoperiod can be condensed into a few months to stimulate gonadal growth and spawning. The change in the direction of the photoperiod (from short days to long days and vice versa) is more important than the actual day length. Exposure of rainbow trout, which normally spawn in late autumn, to long days early in the cycle followed by short days in late spring will advance spawning time by 3–4 months.

Oestradiol acts by positive feedback to increase the production of gonadotrophins by the pituitary. Injection of testosterone, which is aromatized to oestradiol, has been used to increase the production of gonadotrophins. Oestradiol is also a potent inducer of vitellogenesis in the liver, which provides the components for yolk in the growing oocytes.

In addition to environmental cues, spawning can be induced by treatment with a crude preparation of

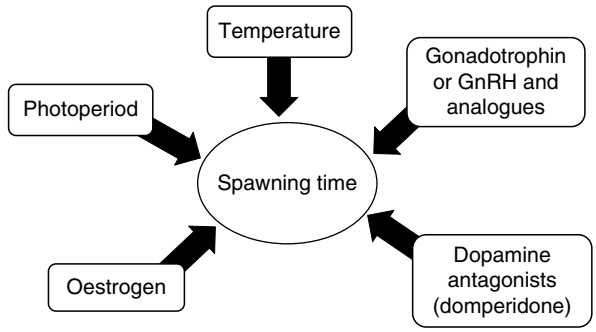


Fig. 5.23. Environmental and hormonal effects on spawning.

fish gonadotrophin or hCG (available as Chorulon® from Intervet), but species-specific preparations of gonadotrophins may be needed in some cases. Thyroid hormone also acts in synergy with gonadotrophin to promote the final maturation of oocytes. GnRH can also be used to induce spawning and it has several advantages over gonadotrophins, since it is an easily synthesized small molecule. More active GnRH analogues have also been developed and several slow-release delivery systems are being investigated. These compounds are also orally active but the dose required is ten times higher than that required by injection. The most potent GnRH analogue is the salmon GnRH analogue [D-Arg6, Pro9 NEt]-sGnRH (sGnRH-A), although analogues of mammalian GnRH (mGnRH) such as [D-Ala6, Pro9 NEt]-mGnRH (GnRH-A) are also very active. The GnRH-A approved for aquaculture is azyGlynafarelin, available as Gonazon® from Intervet. The increased activity of these analogues comes from a combination of increased binding affinity to receptors on pituitary gonadotrophs and increased hydrophobicity and resistance to enzymatic degradation.

Treatment with dopamine receptor antagonists, such as domperidone, in combination with GnRH-A improves the induction of spawning in some species, such as salmonids, but not in others, such as sciaenids. Syndel Laboratories (Vancouver, Canada) has marketed a formulation of sGnRH-A and domperidone under the name Ovaprim®.

A variety of hormone-delivery systems for long-term administration of GnRH-A to improve spawning have been developed. They include implants of pellets of cholesterol or ethylene vinyl acetate, biodegradable microspheres using co-polymers of lactic acid/glycolic acid or fatty acid dimer/sebacic acid. Depending on the preparation, these can release GnRH-A for 1–5 weeks.

To synchronize oocyte maturation and ovulation in fish with synchronous oogenesis, two injections of GnRH-A (10–100 μg kg^{-1}) are given 3 days apart, or a single implant (10–50 μg kg^{-1}) is used. Alternatively, a priming dose of 5–10%, followed by a resolving dose of 90–95% along with a dopamine antagonist, is used. In fish with asynchronous ovarian development, GnRH-A delivery systems are preferred to injections of GnRH-A.

For more information on sex differentiation and the manipulation of spawning in fish, see the reviews by Rajendiran *et al.* (2021) and Baroiller and D'Cotta (2016).

Endocrine effects on growth and nutrient utilization

Increasing growth rate and feed efficiency is particularly important in aquaculture, due to the long production cycle of cold-water fish. The physiological functions of fish are dramatically affected by environmental factors, particularly water temperature, since fish are poikilothermic animals. The production cycle normally lasts for years, with a consequently greater risk of loss of fish from disease or accidental release. In contrast, production cycles for poultry, pigs and cattle are much shorter. Feed costs account for 50% or more of the cost of production in aquaculture, so improvements in feed efficiency can dramatically reduce operating costs.

Various dietary components can have dramatic effects on hormone levels but it is difficult to measure these effects, since changes in one component of the diet are often linked to changes in other dietary components. Hormones regulating growth and appetite are regulated both by internal signals which reflect the metabolic status of the organism and by external environmental signals such as food availability and composition of macronutrients (protein, carbohydrate and lipid) and micronutrients in the diet. Endocrine systems can be regulated at a number of points, including neuroendocrine signalling, synthesis and secretion of the hormone, transport in the blood, metabolism in peripheral tissues, receptor binding, and metabolism and clearance of the hormone (see Chapter 1).

In all vertebrates, feed intake is regulated by orexigenic and anorexigenic endocrine factors which act on feeding centres in the brain (see Section 3.10). Several homologues of mammalian appetite-regulating peptides have been characterized in fish. NPY has been shown to regulate both appetite and digestive function in fish, either in response to availability of feed or during natural periods of food deprivation. Orexins appear to be important as a long-term regulator of feed intake, while ghrelin acts as a short-term hunger factor. The anorexigenic neuropeptides cocaine- and amphetamine-related transcript (CART) and gastrin-releasing peptide (GRP) may also be involved in the long-term regulation of feeding. However, differences in habitats, feeding habits and responses to fasting among different fish species suggest that the endocrine regulation of feeding is somewhat species specific. CART may be involved in increasing feed intake with increased

temperature in Atlantic cod, while neuropeptide Y (NPY) may regulate seasonal changes in feed intake in winter flounder. For more information, see Bertucci *et al*. (2019).

Steroid hormones, particularly androgens, can increase growth rate but they are not used commercially. Thyroid hormones are important in larval development in vertebrates. The yolk of newly fertilized eggs from salmonids and striped bass contains high levels of thyroid hormones, and supplementing eggs or larvae with additional T_3 improves growth and survival of the larvae and fingerlings. Thyroid hormones are involved in the regulation of growth and energy utilization and they may play a permissive role in growth to potentiate the anabolic effects of insulin and growth hormone. Food deprivation decreases the levels of thyroid hormones and reduces the conversion of T_4 to T_3. Dietary protein and carbohydrate affect thyroid hormone production. Carbohydrate stimulates the secretion of T_4, while protein increases the conversion of T_4 to T_3. Restricted growth rate seen at high stocking densities is accompanied by decreased levels of plasma thyroid hormones.

Fish growth follows a pattern that is related to day length. Long day length stimulates growth, in part because the fish has more time to forage for food but also due to melatonin production by the pineal gland. Melatonin production is regulated by exposure to light and can affect the production of growth hormone (ST) and thyroid hormone metabolism. This affects the rate of growth and sexual maturation.

ST and insulin-like growth factors I and II (IGF-1 and IGF-II) are the most potent growth promoters in teleost fish. They act on target tissues, including muscle and bones, in synergy with androgens and thyroid hormones to increase growth. In fish, ST is involved in almost all physiological processes, including osmotic balance, lipid, protein and carbohydrate metabolism, reproduction and growth. ST release from the pituitary is increased during food deprivation or when low-protein diets are fed, and this is thought to be due to direct effects of glucose and amino acids on the pituitary. However, in these situations IGF levels decline, so ST directly stimulates lipolysis. These changes are reversed when feed is available and IGF levels increase to promote growth.

Exogenous recombinant ST from chickens or cattle injected into salmon improves growth and feed conversion efficiency. ST analogues have also been developed that have increased potency and stability; these are also active orally in fish but the dose required is higher than that given by injection. Immersing trout fry for 30 min in a solution of homologous ST also dramatically improved growth. Transgenic salmon and Arctic charr have also been produced that express high levels of ST and they have a dramatically increased growth rate. The AquAdvantage salmon was approved by the US Food and Drug Administration (FDA) in 2015 and by the Department of Environment and Climate Change Canada in 2017. For further information on genetic manipulations in aquaculture, see Wang *et al*. (2021).

Insulin and glucagon regulate lipid and carbohydrate metabolism as well as growth in fish. The level of insulin is increased in faster-growing fish, but it is not clear whether insulin stimulates growth or whether the increased level of insulin is a consequence of increased feed intake. Under natural photoperiod and temperature changes, fish show an active period of feed intake and growth rate during times of increased temperature and photoperiod. The level of insulin is highest during the times of rapid growth and lowest in coldest weather, when feeding activity is lowest. During short-term food deprivation, glucagon and glucagon-like peptides stimulate gluconeogenesis to maintain blood glucose levels. Injection of glucose into fed brown trout decreases levels of glucagon but does not affect the level of insulin, although it is increased by the amino acid arginine.

Applications

Periods of feed restriction or 'nutritional stress' normally occur in wild populations and this can be used to increase feed conversion efficiency and lean growth. Lipolysis and gluconeogenesis are increased during feed restriction as the level of ST increases and insulin and thyroid hormones are decreased. After feed restriction, animals enter a phase of compensatory growth, when levels of anabolic hormones are increased to promote somatic growth rather than storage of energy reserves as fat. This results in improved lean growth and feed efficiency and decreased fat deposition. Compensatory growth after feed restriction is also seen in other species, such as poultry.

Levels of many hormones in fish change with a circadian rhythm. The circadian rhythm of hormone release can also vary with an annual cycle and be

dramatically different in winter than in summer. The release of metabolism-related hormones, such as insulin, is also affected by the composition of the diet, including the quality and proportions of protein, carbohydrate, fats and vitamins and minerals. The time of feeding, as well as the amount of feed given, can affect the amplitude of the daily rhythm of thyroid hormone, particularly T_4, as well as insulin and ST. The time of day when fish are fed can thus dramatically affect growth and feed conversion efficiency, due to differences in levels of hormones. It is therefore advantageous to determine when the hormone levels in the fish are optimal for growth and how different dietary nutrients affect hormone levels. This will allow the development of feeding strategies to promote anabolic hormone production. The goal is to feed fish at the optimal feeding rate and time of day to provide nutrients when the animal is physiologically best able to deal with them. This approach will achieve the best feed conversion efficiency and growth rate. However, in a practical commercial setting, it may be impossible to feed all fish at the optimal rate and time of day. For more information, see the review by Bertucci *et al.* (2019).

Effects of stress on reproduction, growth and immunity

As with all commercial animal production, poor husbandry practices and suboptimal environmental conditions can cause increased levels of stress. In fish culture conditions, crowding is one of the most common sources of stress. Long-term stress can reduce growth performance and reproductive success and increase the susceptibility to disease (Fig. 5.24). Fish respond to increased stress by activation of the hypothalamic–sympathetic chromaffin (HSC) system, to produce catecholamines, and the hypothalamic–pituitary–interrenal (HPI) axis, to produce glucocorticoids. These responses are similar to those described for other commercial animal species (see Section 6.3). Chronic stress reduces the production of sex steroids and impairs gonadal growth, gamete viability and reproductive performance. Levels of ST are increased by stress, but growth rate is decreased due to decreased sensitivity of the target tissues. Chronic stress can also decrease the levels of thyroid hormones and reduce feeding activity, which, together with the catabolic effects of corticosteroids, decreases growth rate.

Physical stress increases the oxygen demand for fish by more than 50%. This becomes more of a problem at high temperatures, due to lowered oxygen content of the water and the accumulation of toxic levels of ammonia produced as nitrogenous waste and carbon dioxide from respiration. These hypoxic conditions result in respiratory stress, which can result in high mortality rates in extreme situations. The hormonal responses to stress also reduce the ability of the fish to maintain osmoregulation, which causes a loss of electrolytes in freshwater fish and an increase in electrolytes in marine fish.

Stress also increases the susceptibility of fish to a variety of viral, bacterial, fungal and protozoan pathogens. Exposure to temperatures at the lower end of the physiological range can also suppress both cellular and humoral specific immune functions, while temperature changes act as a stressor

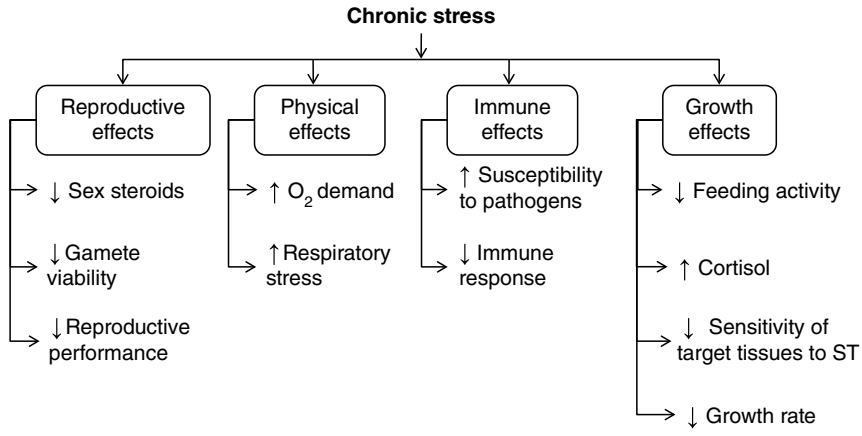


Fig. 5.24. Effects of stress on reproduction, growth and immune response.

to reduce non-specific immune responses. As occurs in mammals, the endocrine system and immune systems in fish interact with each other (see Section 6.3). Cortisol, ST, prolactin, reproductive hormones, melanotrophins and some POMC-related peptides influence immune responses in fish.

Cortisol is the major corticosteroid produced by the interrenal gland in teleost fish. Stress or cortisol administration decreases the resistance of fish to infection by bacteria and fungi. The decreased immune response includes decreased phagocytosis and lymphocyte mitogenesis and decreased activity of antibody-producing cells and levels of IgM. Cortisol is also involved in regulating the ionic balance of fish in seawater.

ST is involved not only in the regulation of somatic growth but also in osmoregulation and gonadal steroidogenesis in fish. Prolactin is also involved in growth and development, osmoregulation and reproduction. Both prolactin and ST are also involved in the stimulation of immune responses in fish. They stimulate lymphocyte maturation and differentiation, activate phagocytes and natural killer cells and stimulate antibody production.

The major steroids produced by the ovaries of female fish are testosterone, oestradiol and androstenedione, while males produce primarily 11-ketotestosterone and 11-β-hydroxytestosterone. Both oestradiol and 11-ketotestosterone decrease lymphocyte proliferation, chemotaxis and phagocytosis. Testosterone reduces the number of antibody-producing cells, as does cortisol, and these steroids act synergistically to inhibit antibody production. In contrast, oestradiol may stimulate antibody production. High levels of the gonadal steroids are present in salmonids during their freshwater migration and maturation and this is linked to immune deficiencies and increased infections during this time.

Skin colour in fish is controlled by the pituitary hormones αMSH and MCH, which act on melanocytes. αMSH darkens the skin by causing dispersion of melanin granules, while MCH concentrates the melanin to decrease skin colour intensity. Both hormones have also been implicated in the regulation of the HPI axis, feeding and osmoregulation in fish. Early studies showed that fish kept in dark tanks are more vulnerable to infections and this is due to the actions of the melanotrophins. αMSH inhibits fever and inflammation by inhibiting the synthesis of the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNFα and stimulating the release of the anti-inflammatory cytokine IL-10. MCH stimulates the proliferation of leucocytes and reduces the inhibitory effects of cortisol on mitogenesis. Other POMC-derived peptides, including ACTH and β-endorphin, have also been implicated in modulating the immune response of fish.

Applications

Although it is impossible to eliminate stress in commercial aquaculture, the negative effects of stress can be minimized. Appropriate stocking densities should be used and water quality should be maintained. Adequate time (days to weeks) should be allowed for recovery from stress from routine procedures such as netting, grading and transporting, and multiple stresses should be avoided. Manipulations should be done in colder water temperatures. Dilute salt solutions can be used for freshwater fish to limit osmoregulation problems. Withdrawal of food for a few days before the stress minimizes contamination of the water from fecal matter and feed and reduces respiratory stress through increased oxygen availability. Anaesthetics (MS222, phenoxyethanol, etomidate) can be used to minimize the stress response.

Several dietary additives have been investigated to reduce the stress response in fish, including amino acids, fatty acids, nucleotides, vitamins and minerals. Important amino acids include arginine for effects on immune response, branched-chain amino acids for effects on protein synthesis, tryptophan and tyrosine for effects on neurotransmitters and methionine for its role in antioxidant and immune status. Vitamins C and E are important for their roles as immunostimulants and antioxidants. Fatty acids such as docosahexaenoic and eicosapentaenoic acids (DHA and EPA) are important as precursors for synthesis of eicosanoids, and marine-origin phospholipid and the carotenoid astaxanthin can also modulate the stress response. Fish fed nucleotide-supplemented diets have enhanced resistance to viral, bacterial and parasitic infections. Organic and inorganic selenium is an essential trace element for fish as a co-factor in the antioxidant enzyme, glutathione peroxidase.

The magnitude of the stress response varies dramatically between different breeds and strains of fish. Genetic selection for fish with reduced cortisol response to stress may alleviate some of the negative effects of stress. For more information on the endocrinology of stress and strategies to relieve stress in fish, see Herrera *et al.* (2019).

Questions for Study and Discussion

Section 5.1 Manipulation of reproduction in mammals

1. Outline the role of endocrine factors in the differentiation of gonadal structures in mammals. What genes are involved in this process?
2. Compare and contrast the process of sexual differentiation in mammals and birds.
3. Describe the sexual dimorphic development of the brain. What factors regulate this process?
4. Describe the differences in the pattern of sexual differentiation in cattle, sheep and pigs.
5. Describe the endocrine factors that regulate meiosis in germ cells in mammals. How does this compare with the control of germ cell maturation in fish?
6. Describe the overall hormonal changes during the different phases of the oestrous cycle.
7. Describe the hormonal regulation of follicular development in cattle. How does this differ from follicular development in birds?
8. Outline the mechanism behind the pre-ovulatory surge in LH.
9. Describe the interaction between the CL and uterus in driving luteolysis.
10. Describe the hormonal changes during pregnancy and parturition.
11. Compare and contrast the regulation of LH release as an animal begins puberty with the annual changes occurring in seasonal-breeding animals.
12. Describe the opportunities for manipulation of the oestrous cycle. What hormone preparations are available for this purpose?
13. Outline methods for detection of oestrus in cattle.
14. Describe methods for synchronizing oestrus in cattle and explain the mechanisms behind these treatments.
15. Describe the mechanism of recognition of pregnancy. Outline potential methods to reduce embryo loss following fertilization.
16. Discuss methods for inducing parturition/abortion.
17. Describe factors that affect the resumption of oestrus postpartum.
18. Outline methods for inducing cycling in seasonal breeders.
19. Discuss the potential for the immunological control of reproduction.

Section 5.2 Endocrine manipulations in aquaculture

1. Describe the direct and indirect methods for the use of hormones in sex reversal of fish. Why would they be used?
2. Discuss methods for manipulating the spawning time of farmed fish. Why are they important?
3. Describe the potential for the hormonal manipulation of growth and nutrient utilization in fish. What non-hormonal alternatives can also be used?
4. Discuss the effects of stress on productivity of farmed fish.

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6 Effects on Animal Behaviour, Health and Welfare

This chapter describes the effects of hormones on several aspects of animal behaviour, health and welfare. The role of prolactin in broodiness in poultry is first covered, followed by a description of pheromones and their applications in both insects and vertebrates. The hormones involved in the stress response and the effects of stress on immune function, reproduction and growth performance are described next. Finally, the effects of endocrine disruptor chemicals on endocrine systems such as androgens, oestrogens and thyroid hormone and the testing procedures that are used to evaluate their effects are covered.

6.1 Control of Broodiness in Poultry

Key concepts

- Broodiness is a natural behaviour that allows the hen to incubate and hatch out a clutch of eggs but this inhibits egg production.
- Increased levels of prolactin in broody hens decrease the secretion of LH, which results in regression of the ovary and the cessation of egg laying.
- Broodiness can be prevented by immunization against prolactin or its releasing hormone VIP and controlled by manipulation of the environment.
- Broodiness is a polygenic trait and QTLs have been identified that may lead to control by genetic selection.

Broodiness, which includes incubation and brooding behaviour, occurs when birds spend an increasing amount of time in the nest to incubate eggs. Food and water consumption decrease dramatically and egg production ceases, due to regression of the ovary and oviduct. Broodiness is decreased in battery-type cages and increased in production systems that allow full nesting behaviour to be expressed, such as modified cages and aviary and free-range systems. Broodiness is a natural behaviour that allows the hen to incubate and hatch out a clutch of eggs and care for the chicks. In the past, good broody behaviour was required to breed the next generation but intensive production systems with artificial insemination have made this unnecessary. Broodiness is a problem in modern production systems, since when a hen becomes broody it ceases to lay eggs, due to the regression of the reproductive system.

Selection of birds for high egg production has resulted in loss of the ability to incubate eggs. The original jungle fowl lays 10–15 eggs per year in the wild, while modern strains produce 300 eggs per year; this would be impossible if they showed any signs of incubation behaviour. Incubation behaviour is practically absent in the high-producing White Leghorn breed, while the ornamental Silkie breed has a high incidence of incubation behaviour. Broodiness is a polygenic trait and a cross between the Silkie and White Leghorn breeds was used to identify quantitative trait loci (QTLs) on chromosomes 1, 5 and 8 related to incubation behaviour. These QTLs include genes of the thyrotrophic axis (*DIO2* and *TSHR*), which suggests that thyroid hormones are involved in the loss of incubation behaviour and the improved egg laying of the White Leghorn breed. *DIO2* and the transthyretin gene (*TTR*) are also down-regulated in the duck hypothalamus during the broody stage. In incubating domestic hens and turkeys, corticosterone is increased and ST and T_3 are depressed. For more information, see Basheer *et al.* (2015).

Altering hormone function can be used to control broodiness (Fig. 6.1). Nesting behaviour begins just before an egg is laid and is stimulated by the interaction of oestrogen and progesterone. After birds have been in lay for a few weeks, nesting behaviour may become prolonged and advance to become broodiness. An extensive brood patch can

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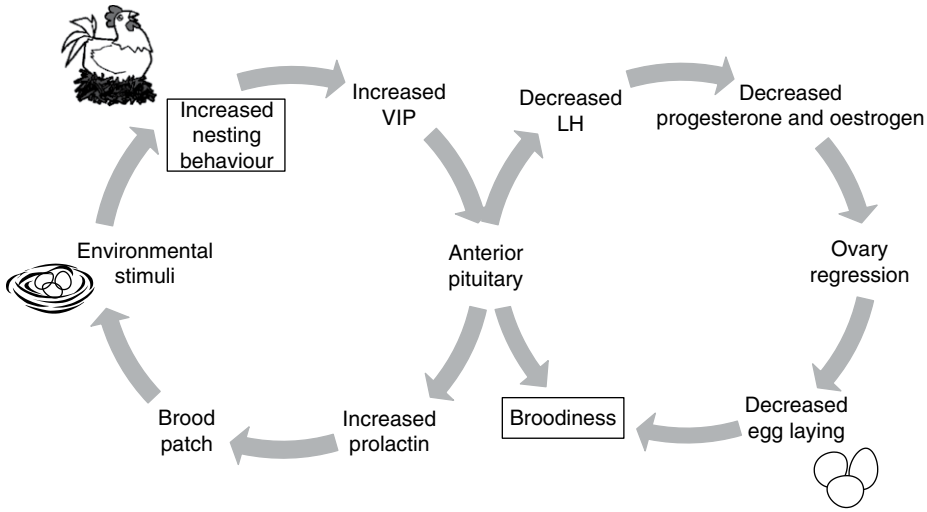


Fig. 6.1. Factors affecting the development of broodiness.

develop on the breast, designed to improve heat transfer from the hen to the developing embryos. Prolactin acts in concert with the ovarian steroids progesterone and oestrogen to increase vascularization and feather loss in the brood patch. Stimulation of the brood patch by the eggs or a familiar nest site maintains the high levels of prolactin release and, once broodiness is established, oestrogen and progesterone are no longer needed. Prolactin also decreases the secretion of LH at the level of the pituitary and through decreased secretion of GnRH-I, which results in regression of the ovary, depressed plasma oestrogen, progesterone and testosterone, and the cessation of egg laying.

The secretion of prolactin by the anterior pituitary gland is controlled by the avian prolactin-releasing hormone, a 28 amino acid peptide originally isolated from the gut and known as vasoactive intestinal peptide (VIP). VIP is released from the POA of the hypothalamus and binds to specific receptors on lactotroph cells in the anterior pituitary to stimulate prolactin release. VIP release is increased by serotonin or dopamine and levels of these neurotransmitters are increased in the hypothalamus of broody birds. Dopamine may also act directly on the anterior pituitary to inhibit prolactin secretion. Prolactin suppresses VIP synthesis and release by negative feeedback via prolactin receptors in basal hypothalamic VIP neurons. For more information on the role of prolactin in birds and mammals, see Stewart and Marshall (2022).

The prolactin receptor (PRLR) gene has been suggested as a candidate gene for the control of broodiness and it has been mapped to the Z chromosome. However, there are no differences in the amounts of the prolactin receptor between White Leghorns, which do not become broody, and bantam hens, which commonly develop broodiness behaviour. It may be that differences in the expression of the various isoforms of the receptor are important. For more information, see Wilkanowska *et al*. (2014).

For interest

A 24 bp insert in the promoter of the prolactin gene is associated with decreased broodiness and lower expression of the prolactin gene. White Leghorns, which do not show broody behaviour, have this insert (Bhattacharya *et al*., 2011). Polymorphisms in the VIP receptor (VIPR-1) are also associated with broodiness (Zhou *et al*., 2008).

Applications

Broodiness occurs where birds are kept on the floor and affects broiler breeders (especially the dwarf strains), egg-layer multiplier flocks and, in particular, turkeys. Broodiness is affected by genotype and is present in most species of laying fowl except White Leghorn. Broodiness can be controlled by

manipulation of the environment to remove the stimuli that encourage nesting behaviour. This includes regular removal of eggs and use of uniform lighting to discourage birds from nesting in dim corners. Adequate numbers of nest boxes should be provided and these should be closed at night. The arrangement or appearance of the nest boxes should also be changed regularly, to prevent the birds from becoming attached to a familiar nest site. Hens that are in the early stage of persistent nesting can be transferred to a pen designed to make nesting uncomfortable, such as one with wire floors and strong updrafts of cold air.

Pharmacological methods to disrupt broodiness have been extensively investigated but no effective practical methods have been developed. Administration of progesterone or oestrogen disrupts broodiness but this delays the return to lay. Treatment with anti-oestrogens (clomiphene citrate and tamoxifen) produced inconsistent results. Further treatments to disrupt broodiness have been directed at either increasing gonadotrophin secretion or decreasing prolactin secretion. Treatment with gonadotrophins returns broody turkeys to lay but is too expensive to be of practical use. Treatment with synthetic GnRH is also impractical, since the anterior pituitary becomes non-responsive to GnRH after prolonged treatment. Treatment with a dopamine receptor blocker, pimozide, does not prevent broodiness. Decreasing the synthesis of serotonin with *p*-chlorophenylalanine (PCPA) decreases the production of prolactin but does not return turkeys quickly back into lay. Use of the dopamine agonist bromocryptine delays the resumption of egg laying.

Passive immunization against prolactin in bantam broody hens results in increased LH. Subsequent cloning of chicken prolactin allowed the active immunization of hens against recombinantly derived prolactin coupled to β-galactosidase. This procedure decreases broodiness without affecting egg production and is more effective if it is initiated before egg laying begins. The amino acid sequences of chicken prolactin and turkey prolactin are practically identical, so this should also be effective in turkeys.

Alternatively, hens can be immunized against VIP. Injection of antibodies against VIP decreases prolactin levels to baseline and decreases broodiness. Active immunization of turkeys with synthetic turkey VIP conjugated to keyhole limpet haemocyanin (KLH) decreases the prolactin level and broodiness. Active immunization protocols to decrease broodiness require regular booster injections at 3–5-week intervals to maintain sufficient antibody titres, making this less practical than good husbandry practices to discourage broody behaviour. For more information, see the review by Sharp (2009).

6.2 Applications of Pheromones

Key concepts

- Semiochemicals are naturally occurring compounds that are used by organisms to perceive and communicate with other organisms and the environment.
- Pheromones are semiochemicals used to stimulate a response in another individual of the same species.
- Signalling pheromones induce behavioural changes, while primer pheromones induce long-term physiological changes in the recipient.
- Pheromones are generally lipophilic, low-molecular-weight, volatile compounds or specific mixtures of compounds.
- Lipocalins are proteins involved in the delivery and detection of pheromones.
- Insects detect pheromones through hair-like projections (sensilla) on the antennae.
- Mammals detect pheromones with odour receptors in the main olfactory epithelium and V1R and V2R receptors in the vomeronasal organ.
- In vertebrates, pheromones regulate oestrous cycling, induce mating behaviours, increase aggression in males and are used to mark territory. Most detailed studies of reproductive pheromones have been done with rodents.
- Sex pheromones have been described for pigs, cattle, sheep, goats, elephants and fish.
- The chemical nature of many more insect pheromones than vertebrate pheromones is known.
- Insect pheromones can be used in pest control, population monitoring, mating disruption and mass trapping and in management of honey bees.
- Some applications of pheromones for reproduction control in mammals have been described.

Introduction

The use of chemical signals is an important method for many diverse species to communicate between

and within a species and between a species and its environment, especially over long distances. An appreciation of the use of chemical signals in animals began in the 19th century but it was not until the 1930s that experimental evidence showed that volatile chemicals were involved. For further information on pheromones, see Wyatt (2017).

Naturally occurring compounds that are used by organisms to perceive and communicate with other organisms and the environment are collectively known as semiochemicals. Insects use semiochemicals to locate a mate, host, or food source, avoid competition, escape natural enemies and to overcome the defence mechanisms of their hosts. Semiochemicals include pheromones, allomones, kairomones and synomones. The word pheromone, first used by Karlson and Lüscher in 1959, is derived from the Greek *pherein*, 'to transfer', and *hormōn*, 'to excite', and refers to a chemical messenger released to the exterior of one individual to stimulate a response in another individual of the same species. Pheromones are thus hormones that are used externally to the animal. Allelochemicals function between members of different species and include allomones, kairomones and synomones. Allomones are emitted by a species to control another species for its advantage. For example, when different species compete for the same resources, repellent allomones emitted by one species can reduce the number of individuals present from another species. Orchids produce chemical mimics of female sex pheromone blends of particular insect species in order to attract the males as pollinators. Kairomones benefit the detecting organism, such as odours from predators and prey. They are thus emitted by a species to its disadvantage and can be due to efforts to overcome stresses. For example, pears release ethyl-2,4-decadienoate, which is a highly potent attractant to the codling moth, *Cydia pomonella*, which is a serious insect pest of walnuts, apples and pears. Synomones are mutually beneficial to both the sender and receiver.

The field of chemical communication and semiochemicals has concentrated mainly on insects, where it is also known as 'chemical ecology', although several vertebrate pheromones have now been identified. Current research on pheromones is involved in structural identification of new semiochemicals, determining receptor function and signal transduction mechanisms and developing natural product analogues.

Types of pheromones

Depending on their effect on the recipient, pheromones are divided into two main types: signalling (or releaser) pheromones and priming pheromones. Signalling or releaser pheromones transfer specific information to induce changes leading to a prompt behavioural response in the recipient. They include sex attractants, which either lead to aggression or are involved in courtship or copulatory behaviour. Alarm pheromones prompt organisms to evacuate an area. Aggregation pheromones bring others to a food source or a suitable habitat, or bring others to a sexual partner. Dispersion pheromones maintain optimal separation between animals and maintain separation between territorial social groups. Priming pheromones induce physiological changes that may have a long-term influence on the recipient, such as the induction of puberty or the termination of anoestrus. Some pheromones, such as queen bee mandibular pheromone, elicit several different responses and this is known as pheromone parsimony.

Chemistry of pheromones and their binding proteins

The chemical structures of several pheromones are known but in most cases the existence of a pheromone is implied from behavioural studies when the chemical structure of the pheromone has not yet been established. Most of the work comes from studies on insects, with much less work on pheromones from vertebrates. Most pheromones are organic compounds or mixtures of organic compounds. They may contain functional groups, such as carbon–carbon double bonds, carbonyl, hydroxyl, carboxyl and ester groups, and may be specific optical isomers (enantiomers). Most volatile pheromones are molecules with 5–20 carbon atoms and include hormones or their breakdown products. They are released in microgram or picogram quantities and therefore can be difficult to identify in a background of hundreds of other compounds. Pheromones can be identified by headspace collection where an air sample is passed over the insect or whole antennae with its sensory organ in an isolated aeration chamber, and the air containing a mixture of volatiles with a positive response is then analysed by gas chromatography. Pheromones are produced by all animals in a group, with some animals producing more than

others and thus eliciting a response. For pheromones that are mixtures of compounds, the concentration of the different components of the mixture can differ among related species. For more information on the comparative structure of vertebrate pheromones, see Apps *et al*. (2015).

Several criteria for identifying pheromones have been suggested (Wyatt, 2017). Pheromones should illicit a unique response and this response should be the same *in vivo* as in a bioassay. The response should occur at natural (i.e. physiological) concentrations and all components of a pheromone mixture are required for the response. The response should also have evolved to fulfil a particular function. Pheromones differ from individual 'signature mixtures' of compounds that are used to identify individuals. Individual recognition is based on learning the different chemical profiles of individuals, allowing familiar and unfamiliar animals to be distinguished. For example, a mother sheep can distinguish her lambs from others, and in social insects such as ants, bees, wasps and termites, animals can identify nest mates and distinguish members of one colony from others.

There are several examples of very similar, or even identical, compounds being active as pheromones in different species. In insects, it is common for closely related species to use similar mixtures of compounds as pheromones, but the ratios of the components are different or other components are included to give species specificity. In other cases, the same pheromone is used for similar species that have different timing of their reproductive cycle, so there is no confusion. In other cases, widely different species utilize the same pheromones. For example, (Z)-7-dodecenyl acetate is a sex attractant for Asian elephants as well as the turnip looper and cabbage looper and many species of butterflies and moths.

A family of low-molecular-weight soluble proteins known as lipocalins (Fig. 6.2) acts as carriers for pheromones and is important for their delivery and detection. Members of the lipocalin family include odorant-binding proteins (OBPs), which are produced by glands in the nasal cavity to concentrate volatile odorant molecules and improve their detection. Major urinary proteins (MUPs) present in the urine and saliva of rodents act to stabilize and control the release of volatile pheromone deposits. Aphrodisin is a pheromone-related protein in the vaginal discharge of hamsters (see below). These lipocalin proteins have a common

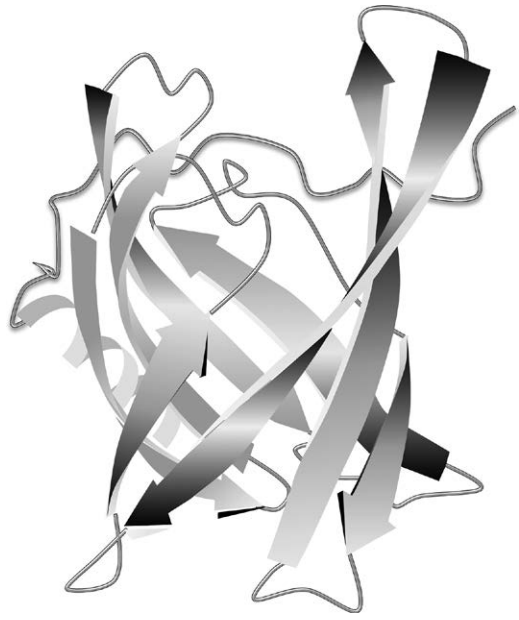


Fig. 6.2. Structure of typical lipocalin (adapted from Schiefner and Skerra, 2015).

three-dimensional structure called a β-barrel, which is made of eight anti-parallel β-sheets enclosing the hydrophobic binding pocket that binds the pheromone. The open end of the cup-like structure has a variable region consisting of four loops, which forms the entrance to the ligand-binding pocket, while the other end is closed by short loops. In addition to binding pheromones, some of the pheromone-binding proteins have been shown to have pheromonal properties of their own, whether or not a volatile pheromone is bound.

Other lipocalins bind and transport a variety of lipophilic or chemically sensitive compounds, such as vitamins, lipid, steroids and other secondary metabolites. The specificity of lipocalins is directed, in part, by the sequence and conformation of the four polypeptide segments that form the opening of the β-barrel. Lipocalins have also been genetically engineered to produce so-called 'anticalins', which bind a variety of small and large molecules in a manner analogous to antibodies. For more information on lipocalins and anticalins, see the review by Schiefner and Skerra (2015).

Pheromone production and release

The communication system for pheromones requires a mechanism for emitting the pheromone,

a medium through which pheromones can be transmitted and a mechanism for receiving the pheromone. Pheromones are usually emitted by glandular organs equipped with specialized structures for their release. In insects, most pheromone glands are composed of groups of modified epidermal cells. The glands may be complex and associated with internal reservoirs. Pheromone production is under hormonal control. A 33 amino acid polypeptide called pheromone biosynthesis activating neuropeptide (PBAN) controls the synthesis of sex pheromone in moths. PBANs belong to a class of peptides known as pyrokinins, which have a common C-terminal sequence of Phe-X-Pro-Arg-Leu-NH_2 (where X = Gly, Ser, Thr or Val), which is required for biological activity. Stimulation of the G protein-coupled PBAN receptor results in increased cyclic adenosine monophosphate (cAMP) and an increase in calcium in the pheromone biosynthetic cells.

Chemical signals can be quite volatile and carried for a long distance in air or water by diffusion and passive transport. These signals would provide an immediate message that would last for a short time. For example, a sex attractant released into the environment would indicate that a member of the opposite sex is available for mating. In insects, wing fanning disperses the highly volatile pheromones. Insects typically release pheromones into the air as pulses. In male sheep and goats, a primer sex pheromone produced in the skin evaporates from the hair or wool. In fish, free steroids are released from the gills or in the urine and many other species secrete pheromones in the urine. Some chemical signals can be persistent, stable and non-volatile. This can occur by binding a volatile compound to a protein (such as MUP in rodents) or using more stable chemical signals of larger molecular weight. These signals would be deposited and remain after the animal has left to provide a signal for the longer term. This would be useful for marking territory or indicating the location of a food source.

Detection of pheromones

The perception of pheromones is through taste and smell olfactory receptors; in insects these are the ionotropic receptors and the odorant receptors (ORs). The ORs detect specific volatile compounds while the ionotropic receptors bind more general odorants and are also involved in taste sensation and in sensing humidity and cool temperature. The olfactory receptors are located on olfactory sensory neurons (OSNs) in hair-like projections (sensilla) in the sensory organs concentrated to the maxillary palps and antennae. Each sensillum contains 1–4 OSNs expressing different receptors, and pheromones diffuse through pores in the sensillum to bind to these receptors. This generates an action potential to convey olfactory input to the primary olfactory centre of the brain to elicit a behavioural response.

These chemosensory systems can be extremely sensitive. For example, the antennae chemoreceptors of the male silkworm moth *Bombyx mori* can detect concentrations of the female sex pheromone bombykol as low as 1 molecule in 10^{17}, in air. This allows a male moth to locate a female that is several miles upwind, at night. Similarly, a steroid that induces moulting in crabs and also acts as a sex attractant is active at 10^{-13} M. Pheromones are active over a limited concentration range and are ineffective when the levels are too high. Once the pheromone has been detected, it is inactivated, so that the animal can continue to respond to new pheromone molecules.

Electrophysiological recordings from whole antennae have been used as a bioassay for insect pheromones. This involves mounting the antennae between two electrodes and recording the electroantennogram, which is the summed electrical response from all the receptors responding to a puff of air containing the pheromone. For further details, see Bohman *et al.* (2018).

In mammals, odorants are detected in the olfactory sensory neurons in the main olfactory epithelium (MOE) that lines the nasal cavity (Fig. 6.3). Each olfactory neuron expresses only one OR, which can recognize either a narrow group of odorants or a wide range of multiple odorants. The detection of odorants involves the combined response of many signals from individual neurons with different receptor molecules. This allows the discrimination of a very large number of odorants. Most mammals also have a second olfactory organ, known as the vomeronasal organ (VNO), which is involved in pheromone detection. The VNO is a tubular cavity in the nasal septum, lined with sensory epithelium and vomeronasal glands, which secrete a fluid into the lumen. The lumen of the VNO is connected to the nasal cavity by narrow ducts, which allows the movement of secretions between the two compartments. During the 'lip curl' Flehmen response in male

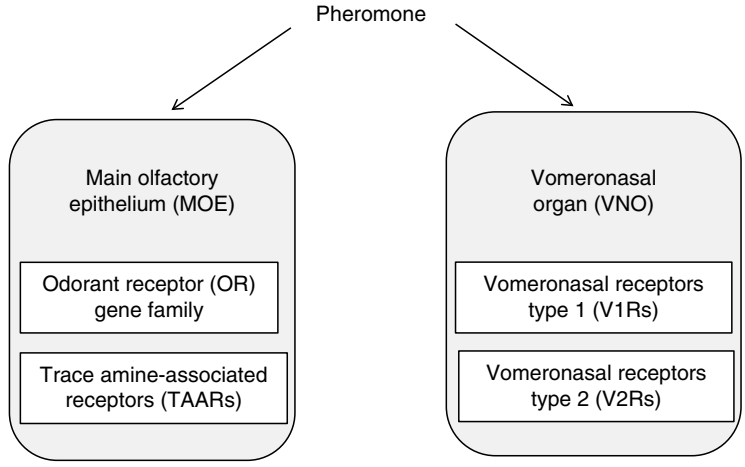


Fig. 6.3. Receptors involved in the detection of pheromones in mammals.

courtship behaviour in certain mammals, including cats, ungulates, bats and marsupials, fluid containing female pheromones is sucked into the VNO and the pheromones are detected. Vomeromodulin, a 70 kDa odorant-binding glycoprotein, is expressed in the VNO and presumed to be involved in the binding of pheromones.

There are two families of OR molecules in the MOE that are all G protein-coupled receptors (GPCRs) (see Section 1.3) and contain the conserved subunit $G_{\alpha olf}$. The OR gene family consists of 800–1500 genes in mammals that code for rhodopsin-like GPCRs that vary dramatically in amino acid sequence and can bind a wide variety of ligands and volatile pheromones. These genes are highly polymorphic and this accounts, in part, for individual differences in the ability to detect specific odours. These receptors are involved in the attraction responses to the male urine thiol, (methylthio)-methylthiol (MTMT), aversion responses to the fox odour, 2,5-dihydro-2,4,5-trimethylthiazole (TMT), and suckling responses to the rabbit mammary pheromone, 2-methylbut-2-enal. A second group of receptors in the MOE, the trace amine-associated receptors (TAARs), are a smaller family of GPCRs that are distantly related to biogenic amine receptors. There are 15 mouse and six human TAARs. Ligands include 2-phenylethylamine, an aversive carnivore odour that activates TAAR4, and trimethylamine, a sexually dimorphic mouse odour that activates TAAR5.

There are also three families of GPCRs that are expressed in the VNO; these are the vomeronasal receptors type 1 and 2 (V1Rs and V2Rs) and the formyl peptide receptors (FPRs). In mice, the V1R family has 187 active members and the V2R family has 121 active members, which are primarily involved in detection of pheromones. In humans, who do not have an active VNO, only five V1R genes are active and no active V2R genes have been found. All the receptors are GPCRs with seven transmembrane domains; V1Rs have a short N-terminal extracellular domain and the V2Rs have a very large N-terminal extracellular domain involved in ligand binding. The V1Rs interact with $G_{\alpha i2}$-coupled protein, while the V2Rs interact with $G_{\alpha o}$-coupled protein. The V1Rs and V2Rs only respond to individual pheromones. The V1Rs react with lipophilic and volatile odorants, including sulfated oestrogen, androgen, corticosterone and pregnane metabolites, while the V2Rs react with water-soluble proteins, such as MUPs, major histocompatibility complex (MHC) peptides and exocrine gland-secreting peptides (ESPs). ESPs are secreted by extraorbital, Harderian and submaxillary glands into tears, nasal mucus or saliva of mice and rats but are absent in humans. ESP1 is a sex pheromone produced by males, while ESP36 is female specific. Vomeronasal FPRs are expressed in VNO sensory neurons that do not express V1Rs or V2Rs. In most mammals, FPRs function in the immune system to recognize formylated peptides secreted by bacteria or mitochondria during infection or tissue damage and in the VNO they respond to metabolites produced by endogenous, invasive or environmental microbes.

The activation of specific receptor molecules initiates the endocrine and behavioural response to

pheromones such as mating, aggression and fear; they affect hormone levels related to puberty or oestrus and are involved in recognition of individuals. This mechanism provides for the specific recognition of a defined number of pheromones.

In mammals, ligand-binding to ORs stimulates adenylyl cyclase to increase cAMP and opens cyclic nucleotide-gated cation channels, causing membrane depolarization and generating an action potential in the neuron. Alternatively, a second pathway exists for V1R and V2R receptors and ion channels that is similar to bitter and sweet taste transduction mechanisms. In this system, phospholipase C is activated to increase IP_3 and trigger the opening of an ion-channel transient receptor potential channel 2 (TRPC2), causing an action potential (see Section 1.3).

Insect pheromones penetrate the cuticle of the antennae sensilla through wall pores and are bound by specific pheromone-binding proteins. These interact with a sensory neuron membrane protein 1 (SNMP1) that mediates the release of the pheromone from the binding protein and its transfer and binding to the receptor. These receptors are heterodimeric complexes consisting of two subunits: a highly conserved OR co-receptor (ORco), which is found in all OR complexes; and a highly divergent PRx subunit that binds specific pheromones. Binding of the pheromone opens the channel complex, leading to an influx of cations into the cell and generating an action potential. This has been proposed to occur directly via the PRx/ORco complex, where ligand binding increases cAMP, which stimulates formation of a cationic pore within PRx/ORco. A second proposed mechanism is that ligand binding stimulates a G protein ($G_{\alpha q}$)/phospholipase C signalling pathway to open calcium-activated cation channels. Phosphorylation of the receptors and high levels of cyclic guanosine monophosphate (cGMP) reduce the signal transduction mechanism. For more information on odour and pheromone receptors, see Silva and Antunes (2017), Fleischer and Krieger (2018), Renou and Anton (2020) and Wicher and Miazzi (2021).

Vertebrate pheromones

In vertebrates, various bodily secretions, such as vaginal secretions and urine, have pheromonal effects. Pheromones have been implicated as indicators of the reproductive status of females and in the induction of behaviours resulting in mating, in aggressive behaviour towards intruder males, in the synchronization of oestrous cycling in group-housed females and in the induction of oestrus by exposure to males. An individual or group can use pheromones or chemical signals to mark its territory. These signals are deposited from urine, or from specialized anal or suprapubic glands.

There is also evidence that individuals can be identified based on odour that is directly related to the composition of the MHC genes in individuals. Odorants may be bound directly by proteins encoded by MHC genes, released into serum and concentrated in urine. It is possible that recognition of MHC types may be used to prevent inbreeding in communal populations. MHC peptides induce the Bruce effect, in which pregnancy is terminated in rodents by exposure of the female to a male that is genetically different from the inseminating male.

Due to the flexibility of mammalian behaviour and its dependence on many factors, behavioural responses to olfactory signals may be less obvious in mammals than in insects. For example, an animal could respond quite differently to a pheromonal signal, depending on its emotional state. Various tactile, visual or auditory cues from the male or female may also affect the response. Repeated exposure to the pheromone can cause the animal to become habituated and then not respond to the pheromone. These factors can make the identification of the pheromone, and the role that pheromones play in vertebrate behaviour, very difficult to determine.

Rodents

Most detailed studies of reproductive pheromones have been done with rodents. The female Syrian golden hamster in oestrus leaves a pheromone trail to attract a male. The attractant pheromone is dimethyl disulfide, but the later stages of courtship and copulation are stimulated by a protein pheromone known as aphrodisin, which is a member of the lipocalin family. Aphrodisin, present in the vaginal secretions of the female, is licked by the male and detected in the vomeronasal organ.

The MUP in the mouse is another member of the lipocalin family and is analogous to α-2u globulin in the rat. MUP is thought to function in pheromone transport in male urine but also has pheromonal properties of its own. In fact, the hexapeptide N-Glu-Glu-Ala-Arg-Ser-Met, which is a truncated form of MUP, accelerates puberty in female mice.

Many polymorphic variants of MUPs are expressed in the liver, enter the bloodstream and are excreted in the urine. The pattern of MUP variants plays a direct role in individual recognition in males. It has also been suggested that MUP1 regulates glucose and lipid metabolism in mice. Male mouse urine contains *sec*-butyldihydrothiazole and dehydro-*exo*-brevicomin (Fig. 6.4) bound to MUP, and these compounds signal aggression in other males, female attraction, and synchronization and acceleration of oestrus. The pheromonal property of these compounds is also illustrated by the fact that a similar compound, *exo*-brevicomin, is a male attractant produced by the female western pine beetle.

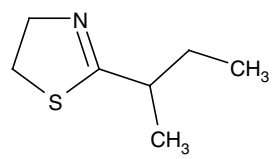
Six small organic compounds that delay the onset of female puberty have also been identified in female mouse urine. Rats produce a maternal pheromone, deoxycholic acid, in the faeces, which attracts the young back to the maternal nest.

Pigs

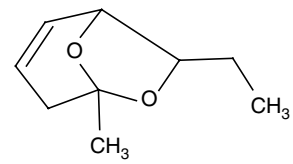
Oestrous sows detect pheromones released by boars. These pheromones are the steroids 5α-androstenone and 3α-androstenol (Fig. 6.5), which are members of the 16-androstene steroid family that is also responsible for boar taint (see Section 3.3). These steroids are synthesized in the testis and subsequently removed from the bloodstream by pheromone-binding proteins in the salivary gland, pheromaxein and salivary lipocalin (SAL). Arousal of the boar results in profuse salivation and release of the pheromones and binding proteins with the saliva. 5α-Androstenone is one of the first vertebrate pheromones available as an aerosol preparation (Boar Mate®).

The boar pheromones cause the sows to 'stand' for mating, so the sow resists forward pressure (lordosis) when in oestrus. These pheromones may also establish dominance hierarchies and decrease fighting of males in regrouping situations. In addition to these signalling actions, boar pheromones have a priming action and increase the onset of puberty in prepubertal gilts. Boar exposure should occur after 140 days of age, since earlier boar exposure actually increases the age at puberty. This may be due to a habituation of the boar presence when the gilt is physiologically unable to respond. Androstenone or boar exposure also decreases the time from weaning to first oestrus in sows.

There is some evidence to suggest that the 16-androstene steroids are active in humans and are found in trace amounts in human sweat. *In vitro* synthesis of the steroids has been demonstrated in human testis microsomes. The 16-androstene steroids have also been found in various tissues in camels. Androstenone is also found in truffles, and pigs have traditionally been used to

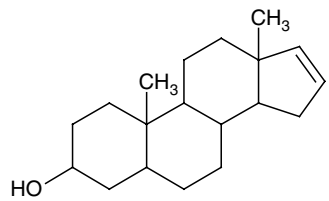


2-*sec*-Butyldihydrothiazole

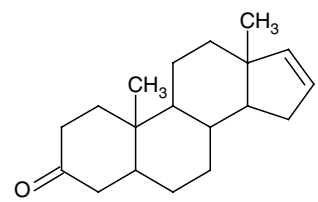


2,3-Dehydro-*exo*-brevicomin

Fig. 6.4. Pheromonal compounds in male mouse urine.



Androstenol



Androstenone

Fig. 6.5. Boar pheromones.

hunt for these underground fungi. Androstenone is also present in caviar, celery and young parsnips.

There is some evidence that pheromones that signal alarm are released in the urine of pigs undergoing stress but the chemical nature of these pheromones has not been investigated. Milking sows produce a maternal pheromone in the skin that stimulates feeding behaviour and post-weaning weight gain in piglets. It is composed of a mixture of six different fatty acids: palmitic acid, 35%; oleic acid, 26%; linoleic acid, 22%; lauric acid, 8%; myristic acid, 7%; and capric acid, 2%.

Cattle

The cervico-vaginal mucus and urine from oestrous cows stimulate sexual activity in bulls. Higher levels of *n*-propyl-phthalate and 1-iodoundecane have been found in urine from oestrous cows, but these compounds have not been proven to act as pheromones. Trimethylamine in the saliva has been suggested as an oestrus-specific signal that stimulates sexual activity in bulls. Detection of olfactory cues as a method for detecting oestrus in cows would benefit the dairy and beef industries (see Section 5.1). The olfactory cues could potentially be detected using trained animals (e.g. sniffer dogs) and electronic sensors have been used experimentally for this purpose. There is also evidence that cows produce pheromones that regulate the oestrous cycle of other cows.

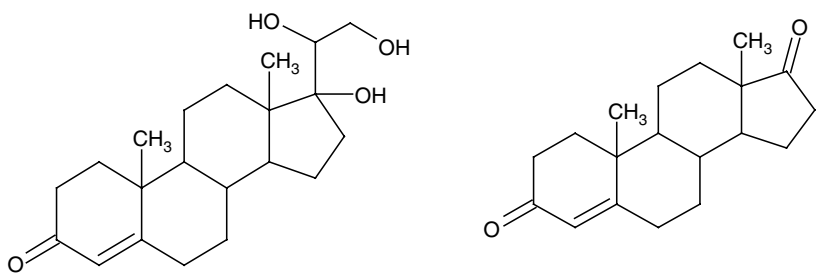
Exposure to bull urine has been shown to accelerate puberty and shorten the calving season in heifers. Bull pheromones could therefore be used to increase the pregnancy rate of first-season heifers and concentrate the calving season in beef heifers. Exposure of cows to a bull also decreases the time to conception after calving.

Sheep and goats

Rams sniff odours from vaginal secretions to detect oestrus in ewes. Oestrous ewes also seek out rams, suggesting that rams emit a signalling pheromone. The introduction of a male also aids in the termination of seasonal anoestrus and synchronization of oestrus in sheep and goats. A primer pheromone is produced in the sebaceous glands of male sheep and goats; the production of this pheromone is induced by testosterone. The pheromone is released from the male hair or wool and stimulates the neuroendocrine system in females to increase the frequency of pulsatile LH release and induce ovarian function and cycling. The compounds 1, 2-hexadecanediol and 1, 2-octadecanediol appear to be responsible for this pheromonal effect in sheep.

Fish

The existence of chemical signals for fish has been demonstrated since 1932, but the compounds responsible have only been identified more recently. Most of the work has been done with goldfish as a model system. About 10 h before spawning, female goldfish produce large quantities of di- and trihydroxy-4-pregnen-3-one, testosterone and 4-androsten-3,17-dione (Fig. 6.6). These steroids act first as hormones to promote oocyte maturation in the female and then are released into the water to act as primer pheromones, increasing the production of gonadotrophins in males. This stimulates the formation of milt (sperm and seminal fluid) and



17α,20β,21-tri-hydroxy-4-pregnen-3-one

4-Androsten-3,17-dione

Fig. 6.6. Primer pheromones produced by female goldfish.

increases aggression in males. The steroids are released as conjugates with glucuronide and sulfate in the urine (see Section 1.2) and free steroids are released from the gills.

Two steroid glucuronates (5β-pregnane-3α,17α,20β-triol,3-glucuronate and its 20α-epimer) are produced in large quantities in the urine of dominant male tilapia to deter subordinate males, attract females and prime ovulation in females. They stimulate production and release of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (Fig. 6.6), the major oocyte maturation-inducing hormone in teleost fishes, to accelerate oocyte maturation and possibly promote spawning synchrony.

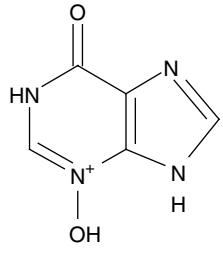
Prostaglandin $F_{2\alpha}$ and its metabolite 15-keto-$PGF_{2\alpha}$ are associated with follicular rupture and ovulation in females. When they are released into the water, they stimulate aggression among males and courtship behaviour towards females. This is an unusual application for prostaglandins, as they normally have only local hormonal effects. Thus, for fish the distinction between hormones and pheromones is very blurred.

Injured fish release an alarm pheromone to trigger anti-predator behaviour in other fish. In ostariophysian (order Cypriniformes) fish, the alarm pheromone has been proposed to be hypoxanthine-3N-oxide (Fig. 6.7), along with oligosaccharides of chondroitin-4-sulfate and chondroitin-6-sulfate which are produced by alarm system cells in the skin. Nothing is known about the chemical structure of alarm pheromones in non-ostariophysian fishes.

Other

Male elephants in musth, when they are highly aggressive, produce the pheromone frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane), which is released in temporal gland secretions, urine and breath. This induces avoidance behaviour in sub-adult males and mating stance behaviours in females in the follicular phase of the reproductive cycle. Female Asian elephants release (Z)-7-dodecenyl acetate in their urine as a signal to males that they are ready to mate. Female garter snakes use a mixture of long-chain fatty acids, ranging from 29 to 37 carbon atoms, as mating pheromone. Squalene is used as a male recognition pheromone in garter snakes. The foul-smelling musk of skunks is produced by scent glands near the anus. The active ingredients are *trans*-2-butene-1-thiol, 3-methyl-1-butanethiol and *trans*-2-butenyl methyl disulfide.

Nepetalactone is the active component of catnip that induces behaviour similar to the mating ritual in domestic cats, lions and tigers. Felinine is a branched-chain sulfur amino acid that is secreted in high levels (3.6 g l^{-1}) in the urine of intact male cats and is a putative pheromone precursor. A synthetic feline facial pheromone product (Feliway®) has been marketed. It is claimed to reduce stress in cats and thus reduce the urine spraying and territorial marking in this species. For more information on mammalian pheromones, see Liberles (2014).

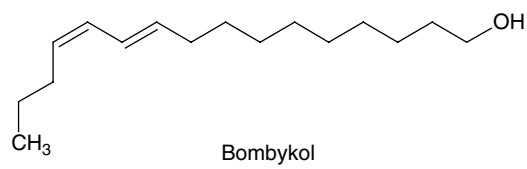


Hypoxanthine-3N-oxide

Fig. 6.7. A major component of the alarm pheromone in ostariophysian fish.

Insect pheromones

Much more is known about the chemical nature of insect pheromones than pheromones in vertebrates. Bombykol (Fig. 6.8), the sex attractant produced by the female silkworm, was the first pheromone to be chemically identified by A. Butenandt and co-workers in 1959. The limited equipment and analytical techniques available at that time made this a very difficult task. The milligram quantities of pure material required for structural determination were purified from half a million scent glands of female silk moths. Sensitive bioassays (see Section 2.2) using the wing fluttering response of the male silk moth needed to be developed to guide the purification work, so the structural identification took over



Bombykol

Fig. 6.8. Sex pheromone of the female silkworm.

20 years to accomplish. With the technology available today, we can analyse the secretion of a single female moth by gas chromatography and use electrophysiological recordings from a male moth's antenna to detect the active components of the pheromone blend. Since this work was completed, the structures of many other insect pheromones have been elucidated. The majority of the lepidopteran female sex pheromones are long-chain alcohols, acetates and aldehydes.

Insects use sex pheromones to attract a mate, aggregation pheromones to indicate to others the site of a good food source, and alarm pheromones to indicate attack by a predator. There are also many other volatile compounds in the environment, including insect–herbivore, host–plant and insect–carnivore semiochemicals that can modify the behaviour and physiology of insects. In some cases, insects do not synthesize pheromones *de novo* but use plant-derived compounds as starting materials for the synthesis. For example, the sex attractant pheromone of danaid butterflies, danaidone, is made by male butterflies from pyrrolizidine alkaloids obtained from plants. Similarly, aggregation pheromones of dark beetles are composed of mixtures of compounds such as *exo*-brevicomin, which are derived from plants they infest.

In eusocial insects (wasps, bees, ants and termites), pheromones are crucial for the establishment of a social hierarchy as well as suppression of reproduction in workers. The queen bee produces queen mandibular pheromone (QMP), which maintains order in the hive, suppresses ovary development in genetically female worker bees to prevent the rearing of new queen bees and stimulates male bees to mate with the queen outside the colony. It also stimulates worker bees to build new comb, rear more bees, forage and store food. Queen bee pheromone is a mixture of several compounds (Fig. 6.9), mainly 9-oxodec-2-enoic acid (9-ODA) and 9-hydroxydec-2-enoic acid (9-HDA). QMP also contains methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA); levels of HOB and HVA differ among different bee species.

Worker bees release an attractant pheromone (Fig. 6.10) from the Nasonov gland to keep the colony of bees together and help disoriented workers find the nest. It is released when swarming or forming a cluster, when marking the location of the nest or marking the location of water. The attractant pheromone comprises seven closely related compounds derived from the essential oils of fragrant

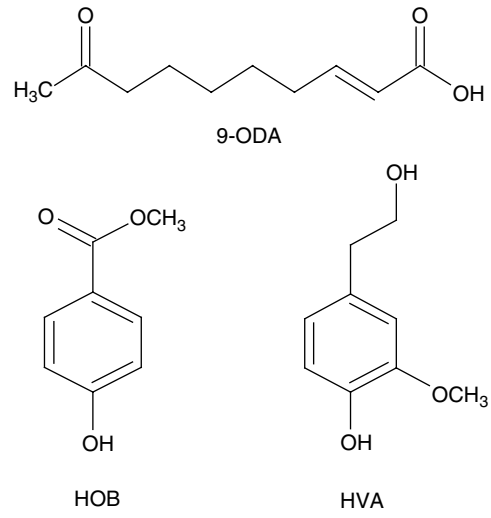


Fig. 6.9. Components of the queen bee pheromone.

plants. The most biologically active of these is citral, which is a component of the fragrance of oranges. Oleic acid from dead and decomposing bees acts as a chemical message to eliminate the dead bee from the hive.

Alarmed bees produce alarm and aggression pheromones to elicit a colony defence response from other workers. A major component of the alarm pheromone is isopentyl acetate, which is a component of the sting material as well as acting as a targeting pheromone to guide other bees to the sting site. 11-Eicosenol also induces stinging and, mixed together with isopentyl acetate, is as effective as natural sting extract in causing a bee to engage in stinging behaviour.

Ants also have alarm and attractant pheromones as well as trail pheromones that they use to mark the trail to a food source. Different species of ants use different chemicals as trail pheromones, which reduces competition for the food. For more information on insect pheromones, see Yew and Chung (2015).

Applications

Pheromone research has applications in pest control, insect management (e.g. honey bees) and in the manipulation of reproduction in domestic animals and humans. Once the chemical composition of a pheromone is known, it can be synthesized chemically in amounts that can be used commercially. For reviews on the use of pheromones and other semiochemicals as biopesticides, see Sharma *et al*. (2019).

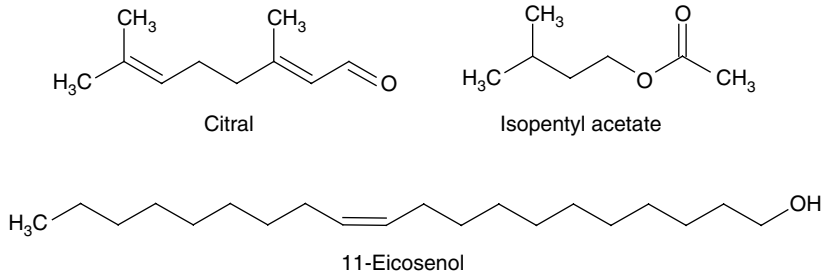


Fig. 6.10. Attractant, alarm and aggression pheromones from bees.

Pest control

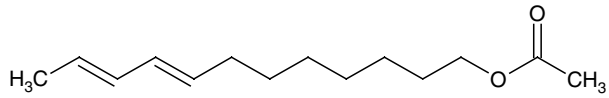
Agricultural damage from insects amounts overall to a loss of about 13% of the potential crop, with much greater losses in some instances. Chemical pesticides account for 99% of the market for insecticides to control insect pests but they can result in water pollution, pesticide residues in food and damage to non-target species, some of which are beneficial for agriculture. Insects also carry viruses, bacteria and parasites that cause diseases such as malaria, bubonic plague, yellow fever, typhus and others. The use of specific pheromonal agents to effectively control targeted insect pests would be of great benefit and is an important part of integrated pest management (IPM) programmes, as they are usually non-toxic and act in a species-specific manner. This requires a controlled-release pheromone dispensing system that mimics the natural pheromone release by insect pests to generate strong plumes of the correct blend of pheromones. SPLAT® (Specialized Pheromone and Lure Application Technology, developed by ISCA Technologies, Inc.) is an amorphous, flowable controlled-release emulsion used to control the release of semiochemicals for insect control. Once the emulsion is applied to a substrate, the aqueous portion evaporates and the active ingredients are then released slowly from the non-aqueous matrix.

In the USA, over 190 active ingredients and 800 formulations of biopesticides have been approved by the US Environmental Protection Agency (EPA). They include: microbial pesticides, in which the active ingredient is a bacterium, fungus, virus or protozoan that is toxic to the insect; botanical insecticides, which are naturally occurring plant-based compounds; and semiochemicals.

Pheromones are natural products and therefore cannot be patented. They are effective in small amounts and individual pheromones are specific for one particular species. This makes pheromones less attractive commercially compared with broad-spectrum chemical insecticides, which are typically patentable and needed in large quantities. Pheromones are generally used as a blend of several chemical components. Some 45 pheromones are listed as biopesticides and these are used for mass trapping, monitoring agents, mating disrupters, aggregation pheromones and as the mite alarm pheromone. Some plant-derived compounds attract insects to a good food source and these can be used together with attractant pheromones from insects.

POPULATION MONITORING The most important use of pheromone-baited traps is for population monitoring. Pheromone-baited traps can be used quantitatively and qualitatively to detect the presence and density of insects. Traps work at low population levels and thus can provide information on whether pests may be infecting an area or a particular crop. This information is then used to regulate the amounts of chemical pesticides that are used and to ensure that they are being used at the most effective times. For example, the pea moth pheromone, dodecadienol acetate (Fig. 6.11), is usually used in traps designed to monitor the size of the moth population, so that conventional insecticides can be used during the vulnerable larval stages. Traps can also be used after spraying to determine whether the treatments were effective. Population monitoring using pheromone-baited traps is also useful for ecological studies and surveys and for quarantine detection of non-indigenous insects.

MATING DISRUPTION Pheromones are used at high concentrations for mating disruption. This high concentration of pheromone can mask the natural trails left by the species and may divert insects from

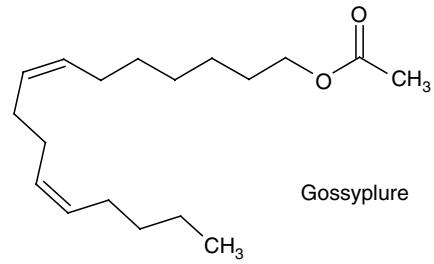


Dodecadienol acetate

Fig. 6.11. Structure of pea moth pheromone.

natural sources, so they are unable to find a mate as effectively. High levels of pheromones may also cause adaptation of antennae receptors and habituation of the CNS, so insects no longer respond to the pheromone. In some instances, an improper blend of compounds in a pheromone mixture or use of a pheromone antagonist can inhibit the response to the natural pheromone. Several pheromone-based products that act as mating disruptors are registered by the US EPA to control 11 lepidopterous pest species. For example, the pink bollworm sex pheromone, gossyplure (Fig. 6.12), is used to disrupt mating. High levels of the pheromone placed indiscriminately make it impossible for the males to follow the pheromone trails left by the females and thus mating is disrupted. While this has been very effective, it is often more costly than broad-spectrum insecticides and can lead to secondary infestations by other insects.

MASS TRAPPING Mass trapping is a powerful and highly specific method for pest control for insects that respond to sex attractants, aggregation signals and oviposition signals for one or both sexes. The appropriate number of traps per unit area must be used to achieve control. Generally, mass trapping is not effective enough to eliminate insect infestations. It is also possible to add an insecticide or biocontrol agent, such as microbials, to the trap to kill the insects that are attracted to the pheromone. In this application a lower concentration of pesticide is needed compared with an insecticide-alone approach. For example, the Japanese beetle pheromone is used in traps designed for lure and kill. In addition to the pheromone, which attracts the male beetles, a floral scent is included to attract the females. These traps are not 100% effective, but they do reduce beetle populations to more acceptable levels. Another example is the use of the aggregation pheromone of the spruce dark beetle (Fig. 6.13) to attract the beetles to about four baited trees per hectare. These trees are then attacked and the heavily infested trees are then



Gossyplure

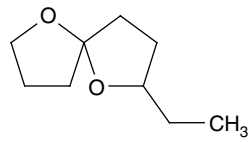
Fig. 6.12. Structure of pink bollworm sex pheromone.

removed and destroyed. Another example is the use of the mite alarm pheromone (Farnesol) along with an insecticide. This reduces feeding of the mites and increases their activity and susceptibility to the insecticide. Synthetic (Z)-9-tricosene (a pheromone that attracts male *Musca domestica* flies) is commonly used as a bait in commercially available housefly traps.

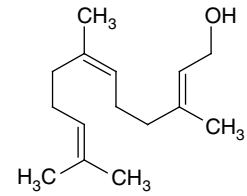
To summarize pheromone use for pest control, pheromones account for much less than 1% of the total pesticide market. They have key roles in pest monitoring systems for specialty crops where the timing of insecticide use is important, as with boring insects. Pheromones have been successfully used for mating disruption, but they are specific for one species and this is a major problem if more than one insect species is involved. Mass trapping is not effective to remove all of the insects and it is usually only the males that are trapped. Aggregation pheromones are useful in specialized areas such as forestry, where part of the crop can be sacrificed for the remainder, but there are not many suitable crops and insect pests for other applications.

Insect management

Pheromones can also be used as part of integrated resource management (IRM) programmes for beneficial organisms. For example, QMP can be used in the management of honey bee colonies. It can be used to create a pseudoqueen when shipping queenless



Spruce dark beetle
pheromone (Chalcogran)



Two-spotted mite
pheromone (Farnesol)

Fig. 6.13. Two pheromones used for mass trapping.

packaged bees and to improve queen-rearing success inside the hive. QMP can also be sprayed on flowering crops (fruit trees) to attract honey bees and improve pollination. The honey bee Nasonov orientation pheromone can be used to reduce the loss of bees due to swarming and to trap bees in food-processing plants and other areas where they are undesirable.

Reproduction control in mammals

Signalling pheromones produced by females can be present at oestrus and pre-oestrus. They can serve as attractants or inducers of sexual activity. Male pheromones can be used to detect oestrus for correct timing of AI to increase conception rates (e.g. Boarmate® in pigs). Primer pheromones can be used to induce puberty in young females, end seasonal anoestrus and improve conception rates (see Section 5.1). In 1956, W.K. Whitten reported the first pheromone effect in mammals and showed that urinary cues from male mice promote oestrus synchronization in group-housed females. Male odours can accelerate the onset of puberty in juvenile females, promoting growth of the uterus and initiating oestrus (the Vandenbergh effect). Groups of females tend to be synchronized with respect to ovulation. This has been known in rodents, humans and also potentially in cattle. Pheromones may have application in contraception and in the treatment of infertility. The 16-androstene steroid 4,16-androstadien-3-one, a component of males' sweat, has been shown to influence brain activity, mood, cortisol response and physiological arousal in heterosexual women. The oestrogen oestra-1,3,4(10),16-tetraen-3-ol, found in female urine, also appears to be a good candidate for a human pheromone. These compounds may have potential to modify endocrine balance in humans.

6.3 Effects of Stress

Key concepts

- Stress refers to a state in which the homeostasis of the animal is threatened or perceived to be threatened.
- Hormonal responses to stress occur via the sympathetic nervous activation of the adrenal medulla (SAM system) to release epinephrine and norepinephrine the nerve fibres of the locus ceruleus in the brainstem to release norepinephrine (sympathoneuronal (SN) system) and the hypothalamic–pituitary–adrenal (HPA) axis.
- Activation of the SAM system provides a rapid hormonal response to support the animal in the 'fight or flight' response.
- CRH stimulates the release of ACTH and glucocorticoids in the HPA axis but also induces anxiety behaviour and reduces feed intake.
- Glucocorticoids increase blood glucose levels, increase protein catabolism, suppress the immune system and have anti-inflammatory effects.
- Stress can be assessed by changes in behaviour and by physiological and endocrine responses. These are more pronounced in chronic stress conditions.
- Stress decreases cell-mediated (Th1) and increases humoral (Th2) immune response.
- Activation of the HPA axis decreases the reproductive hormone axis at several levels.
- Chronic stress decreases growth rate and the efficiency of nutrient utilization for growth and increases energy requirements for maintenance.

What is stress?

A stressor is any environmental change that disrupts homeostasis, and stress refers to a state in which

the homeostasis of the animal is threatened or perceived to be threatened. Eustress results in responses that are either neutral or positive for the animal to make life pleasurable or stimulating and allow it to adapt to the environment within the limits of normality. Distress causes the animal to respond in a way that can negatively affect its well-being or reproduction and which may cause pathological damage. Typical stressors include environmental stress (thermal stress, humidity, noise and light), social stress (mixing, confinement), handling stress (transportation, restraint and castration), metabolic stress (weaning, feed or water restriction) and immunological stress from infectious disease and injury.

The response to a stressor can vary among different animals and involves stress-specific effects on the metabolism or behaviour of the animal, as well as hormonal responses that occur in response to most stressors. The response to stress is a mechanism for coping with threats to the survival and well-being of the animal; this occurs by adjusting several systems within the body to maintain homeostasis and inhibiting systems not essential for the current stress situation, such as reproduction and growth. This adjustment of the set-point to maintain a new physiological equilibrium is known as allostasis. These responses are generally meant to be of a short or limited duration, as in acute stress. In chronic stress, when the response is excessive or inappropriate, these adjustments can fail to compensate for the stress, and pathological changes and damage to the animal can occur, including altered behaviour, decreased immune protection and increased disease susceptibility, or altered metabolism that impacts growth and productivity. For more information on stress and disorders of the stress system, see McEwen and Akil (2020).

Stress can cause changes in behaviour, which may simply involve the animal moving away from a threat. When this is not possible, the animal may fight to remove the threat or try to cope by hiding or developing tonic immobility or other coping behaviours. If behaviour is to be used to assess animal well-being, a thorough knowledge of species-specific behaviours and the normal behaviours of those animals is necessary. Coping or abnormal behaviours are recognized as being not goal oriented and of no obvious benefit to the animal. They include behaviours that are seen in captivity but not in natural settings, such as self-mutilation or repetitive stereotypic behaviours, as well as behaviours that are seen in the natural setting but are performed with abnormal frequency or in an abnormal setting.

Stress cannot be totally avoided in a complex real world. However, not all stress is harmful, and some stimuli are necessary and beneficial to the animal. The well-being of animals in a captive environment can actually be better than in a natural state, since in the natural state predators, starvation, disease and natural disasters can threaten animals. The conditions in the captive environment should be adjusted to maximize the well-being of the animals and not simply to mimic the natural environment.

The effects of stress are ultimately controlled at the level of the hypothalamus and are mediated by changes in behaviour and neuroendocrine effects via the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis. These effects are summarized in Fig. 6.14.

Hormonal responses to stress

Hormone signalling plays a vital role in regulating homeostasis and virtually every endocrine system responds in some manner to specific stressors. This includes hormonal regulation of metabolism, reproduction, growth and immunity. Rapid endocrine responses are mediated by the sympathetic nervous system; this includes the sympatho-adrenomedullary (SAM) axis, which activates the adrenal medulla to produce catecholamines, and the production of norepinephrine by the sympathoneuronal (SN) system. Longer-term effects are due to changes in hypothalamic signalling and pituitary function, resulting in corticosteroid production by the adrenal cortex, via the hypothalamic–pituitary–adrenal (HPA) axis. Stress also decreases the response of the hypothalamic–pituitary–gonadal (HPG) axis to inhibit reproduction (see section below on effects of stress on reproduction). For more information on the neuroendocrine responses to stress, see Godoy *et al*. (2018).

The adrenal gland in mammals consists of two separate organs: the adrenal medulla, on the interior of the gland; and the cortex, which surrounds the medulla on the outside of the gland. The medulla produces the catecholamines epinephrine, norepinephrine and dopamine. The cortex comprises the zona glomerulosa, which produces mineralocorticoids (predominantly aldosterone), and the zona fasciculata and zona reticularis, which produce glucocorticoids and androgens.

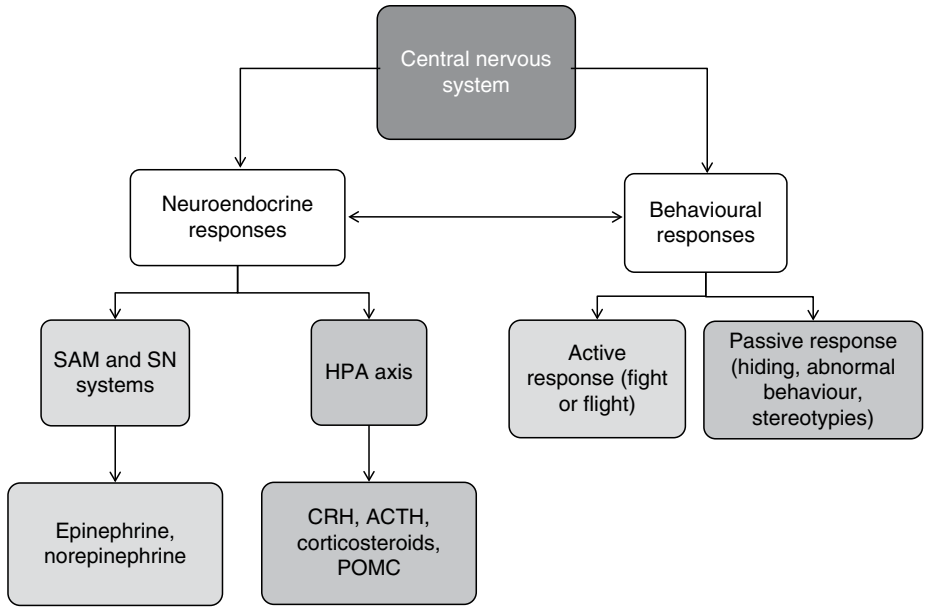


Fig. 6.14. Scheme of overall responses to stress. SAM, sympatho-adrenomedullary system; SN, sympathoneuronal system; HPA, hypothalamic–pituitary–adrenal axis.

SAM and SN systems

Efferent motor neurons carrying information from the central nervous system can be divided into two main systems: the somatic or voluntary system, which controls voluntary movements in skeletal muscle; and the autonomic system, which controls smooth and cardiac muscle and various glands. Neurons of the autonomic nervous system belong to either the sympathetic or the parasympathetic pathway, which generally act in opposition to each other. The neurons of the parasympathetic pathway contact the target organs via cholinergic receptors that use acetylcholine as the neurotransmitter. The sympathetic neurons use norepinephrine as the neurotransmitter at the target organs and thus have adrenergic receptors (see Section 3.7). There are three families of adrenergic receptors: the α1-receptors, the α2-receptors and β-receptors. These are all G protein-coupled receptors (GPCRs), with different signalling cascades (see Section 1.3). The α1-receptors act via a $G_{\alpha q}$ protein to activate phospholipase C (PLC) and increase Ca to activate protein kinase C (PKC). The α2-receptors act via a $G_{\alpha i}$ protein to decrease cAMP and reduce the activity of PKA. The β-receptors act via a $G_{\alpha s}$ protein to increase cAMP to increase PKA and activate the L-type calcium channel. There are a number of selective agonists and antagonists to the different adrenergic receptor subtypes. For more information, see Perez (2020).

The sympathetic and parasympathetic pathways have opposing effects, with the balance between these two systems regulating body systems. The parasympathetic pathway predominates in the relaxed state to lower the heart rate and maintain housekeeping tasks such as digestion. When the animal is threatened, the sympathetic pathway predominates (Fig. 6.15) and stimulates the release of epinephrine and norepinephrine from the adrenal medulla (see Sections 3.7 and 3.12) via the sympatho-adrenomedullary (SAM) system. The locus ceruleus in the brainstem contains nerve fibres that secrete norepinephrine (LUC-NE) that are also stimulated by stress; this is the sympathoneuronal (SN) system. These neurons innervate many areas of the central nervous system to modulate learning and memory, depression and anxiety, sleep and arousal, brain development, motor activity, and sensory information such as pain or touch. Defects in this system are involved in several neurological diseases.

Epinephrine and norepinephrine inhibit the storage of glucose and fatty acids, inhibit protein synthesis and stimulate the release of glucose, amino acids and free fatty acids from muscle, fat tissue and liver.

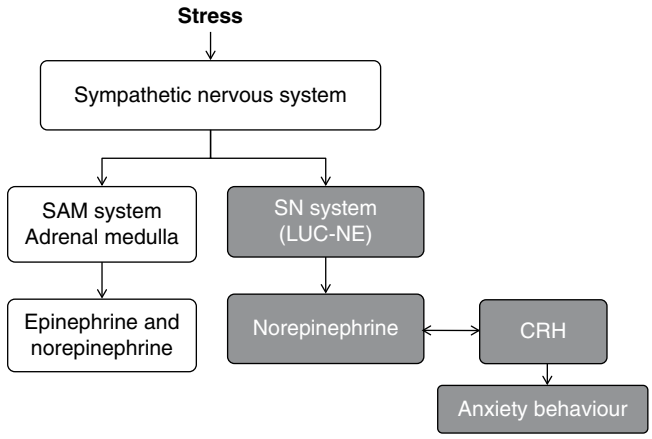


Fig. 6.15. Sympathetic nervous system response to stress.

Heart rate is increased, with blood flow redistributed to the skeletal and heart muscles, and anabolic processes, such as digestion, growth, reproduction and immune function, are suppressed. This is a rapid hormonal response that occurs in seconds to support the animal in the 'fight or flight' response (see Section 3.7). This is followed by a secondary response to activate the HPA axis to release glucocorticoids that peaks with a time lag of tens of minutes.

HPA axis

Neuroendocrine hormones regulate reproduction, shift metabolism, influence growth, affect immunity and alter behaviour. The neuroendocrine HPA axis response involves the release of corticotrophin-releasing hormone (CRH, a 41 amino acid peptide also known as corticotrophin-releasing factor, CRF) by the paraventricular nucleus of the hypothalamus (Fig. 6.16). CRH stimulates the anterior pituitary to release andrenocorticotrophic hormone (ACTH) and subsequently cause the release of glucocorticoids by the zona fasciculata region of the adrenal cortex. Vasopressin (VP) also increases the release of glucocorticoids by potentiating the effects of CRH on the pituitary to increase the release of ACTH and peptides derived from pro-opiomelanocortin (POMC), such as β-endorphin. These opioid peptides have an analgesic effect and reduce the stress response by inhibiting the release of CRH. Oxytocin also exerts a synergistic effect with either CRH or VP on ACTH secretion to increase the release of glucocorticoids. The major glucocorticoid produced in humans, pigs, cattle, sheep and fish is cortisol, while in rats and birds the major glucocorticoid is corticosterone.

Corticosteroids act by negative feedback on the hypothalamus and pituitary to decrease the production of CRH and ACTH. The HPA response occurs more slowly (minutes to hours) and has a more general effect on the animal than the SAM response. ACTH is secreted in a pulsatile manner throughout the day, with higher pulse amplitudes early in the morning, and is thought to enhance attention, motivation, learning and memory retention. Both ACTH and CRH suppress reproduction.

The LUC-NE SN system in the brainstem and spinal cord is also involved in the stress response, as norepinephrine increases general brain activity and stimulates the release of CRH. CRH, in turn, stimulates the release of norepinephrine (Fig. 6.15), which acts both locally and systemically. CRH receptors are present in several areas of the brain involved with cognitive function and emotion. CRH is a major anorexigenic neuropeptide and its secretion is stimulated by neuropeptide Y (NPY) (see Section 3.10), which also inhibits the LUC-NE system. Injection of CRH into the brain of rats produces anxiety behaviour and wakefulness and decreases appetite and sexual receptivity. Elevated levels of CRH in the hypothalamus have been linked to depression in humans. CRH thus acts as a neurotransmitter and plays a role in the behavioural response to stress. The response to CRH depends on previous experience and type of stressor as well as the genetic disposition of the animal.

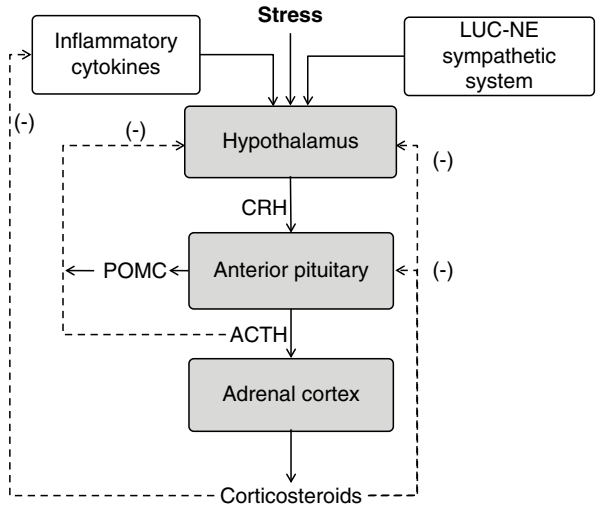


Fig. 6.16. HPA axis response to stress.

CRH AND CRH RECEPTORS The effects of CRH on behaviour can be assessed using CRH receptor antagonists. The first CRH receptor antagonists used were the peptide fragments of CRH, namely α-helical $CRH_{9–41}$ and D-Phe-$CRH_{12–41}$. These peptide antagonists do not cross the blood–brain barrier and had to be injected into the brain to block stress-induced behaviours. CRH is a member of a family of neuropeptides that includes the mammalian CRH analogues urocortin I, II and III, the amphibian peptide sauvagine and urotensin I from fish (Table 6.1 and Fig. 6.17). These peptides may have evolved from an ancestral peptide related to helping organisms deal with environmental changes during their development. The peptide antagonists antisauvagine-30 and astressin have been developed, based on the structure of this family of peptides (Fig. 6.17). A number of non-peptide receptor antagonists that readily cross the blood–brain barrier have also been developed.

CRH acts via two classes of receptors, CRHR1 and CRHR2 (α, β and γ forms), all of which are G protein-coupled receptors (see Section 1.3). There is also a CRH-binding protein (CRHBP) that is thought to regulate the concentration of free extracellular CRH. CRH has 15 times higher affinity for CRHR1 than CRHR2. CRHR1 is linked to increased anxiety-like behaviour and is localized to the brain cortex and cerebellum. CRHR2α is localized to the lateral septum and paraventricular and ventromedial nuclei of the hypothalamus; CRHR2β is found in heart, skeletal muscle and in the choroid plexus and cerebral arterioles in the brain; and CRHR2γ is localized to the amygdala. These receptors have been cloned and expressed in mammalian cells and these cells have been used in an activity-based reporter system to screen for non-peptide antagonists for specific receptors.

Table 6.1. Naturally occurring CRH-like peptides.

Hormone	Species/source	Function
Urotensins	Fish	Ion balance
Sauvagine	Amphibians	Osmoregulation in skin, binds to CRHR1 and CRHR2
Urocortin	Lateral hypothalamus, peripheral tissues (lymphocytes, GI tract)	40-fold higher affinity for CRHR2, decreased gastric emptying and food intake
CRH	Neuropeptide	Release of ACTH by anterior pituitary, behavioural changes

Studies in transgenic mice have linked activation of CRHR2 to reduced signs of anxiety, suggesting that CRHR1 and CRHR2 have opposite effects on stress behaviour. This is important for allostasis, which is the adaptive response to stress by changes in hormones and autonomic responses. CRHR1 present in the pituitary gland is critical for the rapid initiation of corticosteroid release in response to stress, while CRHR2 is thought to be involved in the proper recovery of the response, which may be

Peptide	Sequence
Human CRH	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-NH
Frog Sauvagine	QGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI-NH
Carp Urotensin	NDDPPISIDLTFHLLRNMIEMARNENQREQAGLNRKYLDEV-NH
Human Urocortin	DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV-NH

Fig. 6.17. Amino acid sequence of the CRH family of peptides.

independent of the negative feedback by glucocorticoids. For example, injection of CRH into the brain results in increased blood pressure and heart rate, as is seen during stress. In contrast, CRH or urocortin given systemically causes vasodilation and decreases blood pressure. Urocortin also decreases gastric emptying and food intake (see Section 3.10 for a discussion of factors affecting food intake). Urocortin (I, II and III) is a family of CRH-related peptides produced by the lateral hypothalamus as well as in peripheral tissues, including lymphocytes and the gastrointestinal tract. Urocortins II and III bind CRHR2 with greater affinity than CRHR1 and are thus considered the natural ligands for CRHR2, which dampen the sensitivity to stress. Urocortin I is a high-affinity agonist for both receptors.

Sauvagine (a CRH-like peptide isolated from frog skin) binds to both CRHR1 and CRHR2 receptors. Antisauvagine-30 is a specific peptide antagonist for CRHR2 but not CRHR1. Specific CRHR1 receptor antagonists include NBI 27914, CP 154,526 from Pfizer (Fig. 6.18) and antalarmin, which differs from 154,526 only by a methyl group on the pyrollopyrimidine moiety. These orally active compounds reduce anxiety behaviour, act as antidepressants, decrease the production of stress hormones and can delay parturition. Other antagonists include DMP-696 by Dupont and NBI-30775 from Neurocrine Biosciences, which reduce stress behaviour but do not affect the HPA axis. NBI-30775 has a high affinity for CRHR1 but not CRHR2 and reduces the severity of depression in humans without affecting the production of cortisol, and would therefore be unlikely to cause serious endocrine side effects, such as adrenal insufficiency. This continues to be an important application in human medicine but clinical trials with CRHR1 antagonists have not been very successful. For more information, see Deussing and Chen (2018).

CRH peptides are also produced by and affect immune cells and are thought to act as autocrine/paracrine modulators of inflammatory activity. Antalarmin suppressed the increase in the cytokines

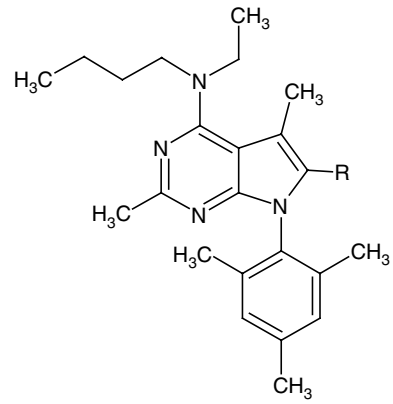


Fig. 6.18. Structure of CRHR1 receptor antagonist. For CP 154,526, R = H; for antalarmin, R = CH_3.

TNF-α, IL-1 and IL-6, which are produced by macrophages in response to challenge with LPS. For more information, see Vasconcelos *et al*. (2020).

Role of various hormones in stress responses

Glucocorticoids are catabolic and stimulate gluconeogenesis in the liver by increasing the synthesis of the enzymes involved in converting amino acids into glucose and increasing the mobilization of amino acids from muscle. Glucocorticoids also decrease the rate of glucose transport and utilization by cells. This increases blood glucose levels by as much as 50% above normal. Cellular protein levels are also decreased by glucocorticoids in all cells except the liver, due to increased protein catabolism and decreased protein synthesis. Although labile proteins are first depleted, prolonged exposure to high levels of glucocorticoids can cause muscle weakness, decreased size of lymphoid tissue and impaired immune function. However, the levels of liver protein and plasma proteins made in the liver do increase as part of the acute-phase response, which occurs in response to infection. Glucocorticoids

increase the mobilization of fatty acids from adipose tissue and increase their utilization for energy. Long-term exposure to high levels of glucocorticoids may suppress growth and accelerate the ageing process and result in metabolic disorders by increasing protein catabolism and causing hyperglycaemia.

Glucocorticoids affect the expression of many genes involving various aspects of metabolism and development. They are permissive for the actions of many hormones, including potentiating the synthesis and actions of catecholamines in increasing hepatic gluconeogenesis. Glucocorticoids have immunosuppressive and anti-inflammatory effects. They also increase gastric secretion, which can lead to the development of gastric ulcers. Glucocorticoids also decrease reproductive efficiency by blocking the effects of LH on the gonads.

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are anabolic hormones that are also produced by the adrenal cortex in response to ACTH and are normally secreted in synchrony with cortisol. However, in critical illness and acute trauma, such as burns, levels of DHEA and DHEAS decrease while the cortisol levels increase. The balance of these steroids after injury affects treatment outcomes, with a high cortisol:DHEAS ratio associated with increased morbidity and mortality, while restoring the ratio improves wound healing, mood, bone remodelling and psychological well-being in older humans. Levels of DHEA but not cortisol decrease with ageing. The stress of increased physical exercise tends to increase levels of DHEA and DHEAS. The role of these steroids in the stress response is not clear but DHEA is thought to enhance immune function and to be correlated with a sense of well-being in humans. For a description of the effects of DHEA on stress, see the review by Bentley *et al*. (2019).

Short-term stress increases the secretion of growth hormone (ST) in non-rodents but reduces the secretion of IGF-1, thus diverting energy from growth to survival. ST acts as an antagonist to insulin in peripheral target tissues to spare blood glucose, while decreased production of IGF-1 reduces growth. Stress also increases levels of IGF-binding proteins. The increased ST may be caused by increased glucocorticoids. The mechanism of decreased IGF-1 during stress is not established, but lower numbers of ST receptors and decreased signal transduction in the liver have been demonstrated during undernutrition or exposure to cold temperature. Under long-term stress, ST release is suppressed.

Levels of prolactin increase within a few minutes in response to acute stimuli and then decrease. Passive, rather than active, coping responses to stress increase prolactin. The increased prolactin may be due to production of β-endorphin, which decreases levels of dopamine, which normally acts as a prolactin-inhabiting factor (PIF) to inhibit prolactin release. Vasoactive intestinal peptide (VIP) and thyrotrophin-releasing hormone (TRH) also directly stimulate prolactin release. The role of prolactin in the stress response is not clear but it has been suggested to modulate immune function.

Acute psychological stress increases thyroid-stimulating hormone (TSH) secretion from the pituitary and increases release of triiodothyronine (T_3) and thyroxine (T_4) from the thyroid in non-rodents, to increase the metabolic rate. Cold temperature increases the activity of thyroid hormones, while chronic undernutrition decreases thyroid activity. This is due to changes in hypothalamic TRH release, pituitary thyrotroph function, thyroid hormone production and peripheral thyroid hormone receptors. The reduction in metabolic rate when food supply is limited would decrease energy usage and improve survivability. Chronic stress reduces thyroid hormone function and elevated levels of glucocorticoids inhibit the conversion of T_4 to T_3.

Renin is released from the kidney by various forms of distress. It acts on angiotensinogen to produce active angiotensin, which is a powerful vasoconstrictor. The activation of α-adrenergic receptors on pancreatic β cells by the sympathetic nervous system also suppresses insulin release. Glucagon release is also increased by aversive states and these effects on insulin and glucagon cause high levels of plasma glucose during stress.

In summary, exposure to distress increases levels of epinephrine, norepinephrine, corticosteroids and, in some species, growth hormone and thyroxine. These hormones have a catabolic function and mobilize energy reserves to allow the animal to deal with the adverse situation. Conversely, levels of anabolic hormones (insulin, androgens and oestrogens) decrease with distress. Prolonged exposure to aversive situations thus results in decreased thyroid activity, reduced body growth and decreased reproductive activity.

Assessment of stress

Behavioural and physiological measures

Animals can respond to stress by changes in behaviour, changes in the autonomic nervous system and

catecholamine production, neuroendocrine changes in the HPA system and changes in metabolism, physiology and immune function (Table 6.2). Not all of these systems are altered by any one stress and there is no non-specific stress response that applies to all stressors. It is thus important to monitor several systems to assess the degree of stress in animals. For further information on the concept of stress and methods to assess stress, see Chen *et al*. (2015).

Animals respond to stress with species-specific behaviours as well as learned behaviours that are specific to the individual animal. Species-specific behaviours have allowed animals to survive in the wild but may result in a harmful response in captive animals and be a threat to welfare when the animal fails to cope with the situation. Individual animals may react differently to the same stimulus, depending on their genetic constitution and previous experiences. Circadian rhythm and environmental effects, as well as age, sex, physiological state and population density, can also affect the reactions of individuals to stress.

Useful behaviours for monitoring well-being include level of activity, posture, vocalization, aggressiveness, movement patterns, feed and water intake and sleep patterns. Animal preference tests, in which an animal can choose between different situations or stimuli, can also be used to assess whether a particular stimulus is undesirable. The amount of effort that an animal is willing to use to avoid the stimulus can be used as a measure of the degree of averseness of the stimulus. In this way, preference testing may be used to rank the suitability of housing systems, food and other environmental factors.

Physiological responses to stress provide the animal with the ability to cope with the stressor. They include increases in heart rate, blood pressure, respiration rate and skin temperature and sources of energy (glucose, amino acids and lipids). In particular, heart rate variability has been proposed as an index of chronic stress but it is difficult to measure. Performance measures of stress include growth, body condition, milk yield and fertility. Immune function is also decreased by stress in order to avoid an excessive immune response. This can be monitored directly (for example, as the neutrophil/lymphocyte ratio; heterophil/lymphocyte ratio in birds) or indirectly, by assessing disease markers, increased positive acute-phase proteins and decreased negative acute-phase proteins. Biochemical measures of energy balance, dehydration and tissue damage (e.g. serum enzymes) can also be used. While it is possible to use these performance and health indicators to assess stress conditions, it is important to use a reference population of comparable genetic merit to compare these indicators.

Table 6.2. Summary of potential indicators for assessing acute and chronic stress (modified from Trevisi and Bertoni, 2009).

Indicators	Acute stress	Chronic stress
Behavioural	Vocalization, restlessness, fight, pain, fear, 'freeze' response	Stereotypies
Physiological	Increase in heart rate, blood pressure, respiration rate, change in skin temperature	Increase in heart rate variability
Performance	Reduced milk yield, milk ejection, altered feed intake and digestion	Reduced growth, body condition score, fertility and longevity; increased metabolic and infectious diseases
Endocrine	Increases in catecholamines, CRH, ACTH, glucocorticoids, oxytocin, vasopressin, β-endorphin, renin and prolactin	Increased basal cortisol, changes after CRH or ACTH challenge, β-endorphin, decreased gonadotrophin and sex steroids
Immune		Increased neutrophil/lymphocyte ratio, monocytes, α-globulin, decreased mononuclear cells after mitogen stimulation, IgM, haemolytic complement Disease markers: increased serum amyloid A, haptoglobin, ceruloplasmin; decreased albumin, lipoprotein, retinol-binding protein
Biochemical	Increased fatty acids and ketone bodies, decreased glucose, dehydration, increased serum enzymes	Prolonged negative energy balance, decreased blood potassium and increased alkaline phosphatase

Hormonal measures

The activity of the HPA axis, as measured by the levels of ACTH and glucocorticoids, has been widely used as an index of stress but changes in levels of these hormones do not necessarily indicate that the animal is in distress. For example, surgical trauma can result in release of ACTH even though the animal is anaesthetized and unaware of what is happening. The magnitude of the ACTH response does not always match the degree of painfulness or unpleasantness of the treatments. Glucocorticoid levels also depend on the behavioural response that occurs due to the stress. Other factors, such as the availability of food and water, infections and stage of lactation, can also affect the magnitude of this response. If an animal perceives an event as threatening, it will respond whether the event is actually threatening or not. Glucocorticoid levels are also increased in situations that are not unpleasant, such as during coitus, voluntary exercise, in anticipation of regular deliveries of food and simply exposure to novel environments. There is a large diurnal variation in the pulsatile release of glucocorticoids, with a peak in humans occurring during the early morning and in rodents in the beginning of the evening. Sampling at the same time every day can minimize this variation.

Repeated regular exposure to a stress, in which the animal can anticipate when the stress will occur, can result in habituation and dampening of the corticosteroid response. For example, electric shocks delivered at irregular intervals produce a higher corticosteroid response than electric shocks given at regular intervals. In the same manner, if the animal has a sense of control and can predict the stress, the glucocorticoid response will be lessened. In contrast, for highly painful and aversive stressors, such as dehorning, the response may increase when the stress is repeated. The endocrine response to stress also depends on the individual coping responses of the animals. If the animal reacts in an active manner in an attempt to control the threat, it will exhibit aggression and status control behaviours. If it is driven by anger, the endocrine responses will be increased levels of norepinephrine and sex steroids, while if it is driven by fear the levels of glucocorticoids and catecholamines will increase and sex steroids will decrease. If the animal reacts in a passive manner due to a loss of control, it will exhibit defeat behaviours, resulting in increased glucocorticoids and decreased sex steroids.

Glucocorticoid levels return to near normal in animals under long-term stress, because of negative feedback of glucocorticoids on ACTH release, but there may be changes in the response of the adrenal glands to ACTH in chronic stress. Repeated challenge with ACTH increases the production of glucocorticoids, so administering ACTH or providing an acute stress to chronically stressed pigs (a challenge test) may result in an increased glucocorticoid production compared with that of non-stressed pigs.

In addition to glucocorticoids, levels of β-endorphin, renin and prolactin are affected by stress, and plasma levels of these hormones have been suggested as possible indicators of animal well-being.

In summary, several different variables should be measured to assess animal well-being accurately, since animals respond in a variety of different ways to aversive situations. Behaviour can be assessed non-invasively and provide information on the nature of the distress and the coping strategy that the animal has adopted. Differences in behaviour among animals kept in different environments may be used to indicate that an environment is causing distress but some changes in behaviour may be difficult to interpret. Additional criteria (Table 6.2) need to be measured to assess the effects of long-term or subtle aversive stimuli.

Issues related to sampling

Measures of physiological or metabolic parameters or endocrine changes in blood samples tend to be invasive and thus induce confounding reactions in the animal. This can be more severe in semi-domesticated or wild animals. Levels of catecholamines can increase within seconds after restraint, while corticosteroid levels increase within a few minutes after restraint and then remain elevated for several hours. Sedating the animals or habituating them to the blood-sampling procedures may reduce the effects of handling but may result in atypical responses. For example, habituation is not suitable for procedures involving the stress of handling. Alternatively, less invasive methods that reduce the degree of handling can be used. Indwelling catheters can be placed in blood vessels and animals can be trained to cooperate with blood-sampling procedures. Remote blood-sampling equipment attached to the free-moving animal can be used for non-invasive blood sampling of large free-range animals. However, implanting catheters requires surgery and it can be difficult to keep the catheters patent.

Alternative non-invasive methods include measurements of cortisol in readily accessible body fluids, such as milk, saliva and urine or in faeces, hair and feathers. Salivary cortisol levels are generally correlated with blood cortisol, but the levels of cortisol in saliva reflect only the free unbound cortisol levels in blood and are only about 10% of the total blood cortisol. Saliva can be collected non-invasively by having the animal chew on a gauze sponge. There can be problems with contamination of the sponges with feed and with rumen fluid in cattle, but this method has been successfully used to assess cortisol status in pigs. Cortisol is also transported from plasma to milk, so milk cortisol concentrations can be used as an indicator of stress in dairy cattle. Urine contains both free cortisol and cortisol metabolites but levels are quite low. There is also a lag time between cortisol release and excretion. For a quantitative estimate of cortisol status, a urine sample should be collected over the entire day. Faecal cortisol may be useful for studies of stress in wild animals but factors such as lag time, volume of faeces and rate of passage need to be considered. Hair and feathers integrate baseline levels and short-term peaks of glucocorticoids over periods up to several months, so glucocorticoid levels in hair and feathers are a useful biomarker of long-term stress in wild and domestic animals.

In plasma, there is an equilibrium between glucocorticoids bound to corticoid-binding protein (CBP, transcortin) or albumin, and free unbound steroids. Only the free steroids are thought to have biological activity, so increasing the concentration of CBP would lower the activity of the glucocorticoids. The ratio of free to bound steroids can be altered by changes in the amount of binding protein, particularly during chronic stress, when protein catabolism occurs. There is, therefore, some controversy over whether the total amount of glucocorticoids or simply the free steroids should be measured to assess the stress response.

Effects of stress on the immune system and disease resistance

Overview of the immune system

The immune system contains several different types of leucocytes (white blood cells) that play integral roles in the overall response to an immune system challenge. This includes granulocytes and monocytes, which are formed in the bone marrow and protect the body by ingesting and destroying invading organisms by the process of phagocytosis. This is called innate immunity (Fig. 6.19). Monocytes mature in tissues to become large macrophages, which can combat foreign pathogens without prior exposure to them through disease or vaccination. The granulocytes include neutrophils, which attack bacteria by phagocytosis, and eosinophils, which attach to parasites to kill them.

Other types of white blood cells include lymphocytes and plasma cells, which are produced in the lymphoid tissues and are involved in the immune reactions. They are involved in acquired immunity (Fig. 6.20), since the response is directed specifically towards foreign agents that have been previously present. Humoral immunity is a type of acquired immunity that refers to the production of antibodies by B-cell lymphocytes (see Section 2.1), while cell-mediated immunity is acquired immunity carried out by activated T cells. There are three groups of T-cell lymphocytes: helper T cells, cytotoxic T cells and suppressor T cells. The helper T cells (Th) produce lymphokines (also known as cytokines), which are peptide hormones and include interleukins (ILs) and interferons (IFNs), which act in a paracrine or autocrine manner to stimulate other immune cells in the process of inflammation and immune reactions. There are two major types of Th cells that are mutually inhibitory. Th-1 cells produce IL-2, IFN-γ and TNF-β to promote cellular immunity and inflammation. Th-1 cells are inhibited by IL-4 and IL-10, which are the major anti-inflammatory cytokines. Th-2 cells secrete IL-4, IL-10 and IL-13 to promote humoral immunity. Th-2 cells are inhibited by IL-12 and IFN-γ, which are the major pro-inflammatory cytokines. Thus, the products of Th-1 cells inhibit the Th-2 cells and vice versa. Cytotoxic killer T cells attack and kill

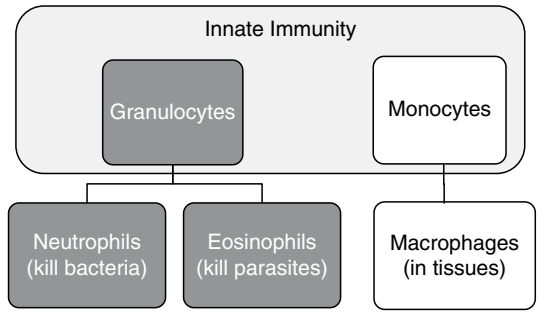


Fig. 6.19. Innate immunity (bone marrow).

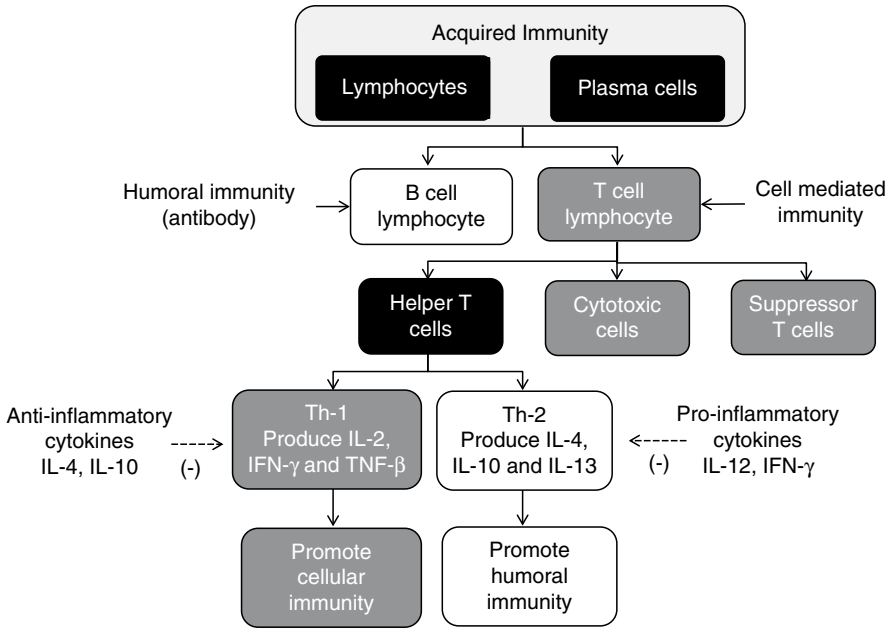


Fig. 6.20. Acquired immunity (lymphoid tissue).

foreign cells or cells that have been invaded by viruses. The suppressor T cells regulate the other cells to prevent severe immune reactions that could damage the animal's own tissues.

Stress from traumatic injury resulting in bacterial or viral infection causes an inflammation response and production of cytokines by macrophages, vessel endothelial cells and fibroblasts. Bacterial infections induce the production of IL-1, IL-6, IL-8 and TNF-α, while viruses induce the production of type I interferons, such as IFN-α and IFN-β. These distinctions are not absolute, but cytokines can be used as markers of infection and for discriminating between bacterial and viral infections.

Cytokines induce a sickness response, causing fever, fatigue, loss of appetite and decreased libido. This response allows the animal to conserve energy and avoid further harm in order to recover, but it may also lead to damaging behavior directed toward other members of its social group, such as tail biting or ear biting in pigs. Cytokines also stimulate an acute-phase response, which includes the production of hepatic acute-phase proteins, including protease inhibitors, coagulation factors, complement factors, transport proteins and scavengers that are released into the circulation. In addition, synthesis of acute-phase-regulated intracellular proteins, including transcription factors, intracellular enzymes and cell surface receptors, is also increased. The role of the acute-phase proteins is to restore homeostasis. For example, C-reactive protein (CRP) (named for its ability to bind the C protein of pneumococci), along with other complement proteins, binds to bacteria in the process of opsonization and tags the bacteria for destruction by macrophages. Tests are available for the rapid determination of CRP in blood and these tests are used to diagnose inflammation and determine the effectiveness of treatments. For more information on the biological significance of cytokines and the various methods of their detection, see Liu *et al.* (2021); and for effects on behaviour, see Nordgreen *et al.* (2020).

Inflammation is the complex of changes that occurs in injured tissue. It is characterized by excess local blood flow, increased permeability of the capillaries, clotting of the fluid in the interstitial spaces and swelling of the tissue. Inflammation serves to wall off the damaged or infected area from the remaining tissues to contain the damage. These changes cause the redness, heat, swelling and pain that are associated with tissue injury. Large numbers of granulocytes and monocytes migrate to the area and are activated by lymphokines and tissue products, such

as histamine, bradykinin, serotonin and prostaglandins, to begin their phagocytic actions.

Stress effects on the immune system

There is a lot of evidence demonstrating that chronic stress from prolonged exposure to aversive conditions reduces the health of animals. Many intensive livestock production systems can reduce the immune response of animals and the resistance to disease. For example, the stress of shipping calves to feedlots increases their susceptibility to bovine respiratory disease (shipping fever). This is due, in part, to the decrease in neutrophil release from bone marrow caused by glucocorticoids. In contrast, shipping pigs decreases body weight but does not reduce immune function, although pigs with lower social status have generally lower immune function compared with dominant pigs. Short-term (acute) stress can enhance the immune function of livestock so that they can better resist a pathogen invasion, while long-term, low-intensity (chronic) stress can cause suppression of the intestinal, cellular and humoral immunities.

The nervous system can directly modulate the activity of the immune system to decrease disease resistance. This occurs by the direct innervation of lymphoid tissue by autonomic nerves, by paracrine action of norepinephrine released from nearby nerves on immune cells and through the HPA axis.

Glucocorticoids affect the immune response in animals to lower the resistance to infection and decrease inflammation (Fig. 6.21). Glucocorticoids affect cell-mediated immunity by decreasing the number of lymphocytes and eosinophils, suppressing natural killer (NK) cell activity and decreasing the amount of lymphatic tissue. Increased levels of glucocorticoids can destroy thymus cells and negatively affect T cells. There are also negative effects on B cells to inhibit antibody formation. Adrenalectomy prevents the negative effects of stress on the immune response in laboratory animals.

Binding of an immunogen such as bacterial lipopolysaccharide (LPS) to toll-like receptors (TLR4) found on the immune cells' surface causes the release of inflammatory cytokines. Glucocorticoids modulate immune responses by inhibiting the production of type 1 pro-inflammatory cytokines, which stimulate cellular immunity. These cytokines, particularly IL-1, also act on the hypothalamus to increase the secretion of CRH, which acts on the pituitary to release ACTH and subsequently increase the production of glucocorticoids from the adrenal cortex. CRH is also produced by immune cells in the spleen or in

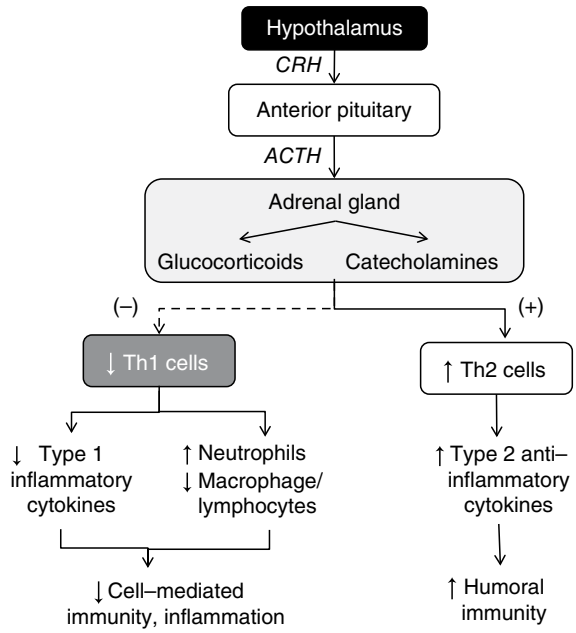


Fig. 6.21. Effects of glucocorticoids and catecholamines on immune function.

nerve endings and acts on immune cells at the site of inflammation. The glucocorticoids and CRH then act by negative feedback on the immune system to increase the formation of neutrophils and decrease the formation of macrophages and lymphocytes. This increase in the ratio of neutrophils to lymphocytes has been suggested as an index of chronic stress in cattle. The production of pro-inflammatory leucotrienes and prostaglandins by immune cells is also decreased and the activity of pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), is inhibited by glucocorticoids.

Both glucocorticoids and catecholamines also increase the production of the type 2 anti-inflammatory cytokines IL-4, IL-10 and IL-13 by Th2 cells. These cytokines promote humoral immunity and protect against extracellular bacteria and parasites and soluble toxins and allergens. Further information on the effects of stress on the immune response can be found in the review by Niu *et al*. (2022).

Thus, cortisol reduces inflammation and speeds up the healing process. In addition to inhibiting the production of cytokines, cortisol stabilizes the lysosomal membranes and reduces the release of proteolytic enzymes from lysosomes. It also decreases the permeability of capillaries and reduces the loss of plasma and the migration and activity of white blood cells. This blocks the further release of inflammatory materials from cells. The reduction in cytokine production also reduces fever. The activation of the HPA axis and the production of glucocorticoids thus keep the immune response in check and prevent it from becoming hypersensitive and therefore harmful.

For interest

Polymorphisms in the human glucocorticoid receptor gene alter the sensitivity of the receptor to glucocorticoids. The ER22/23EK polymorphism caused decreased sensitivity and was associated with improved body condition and longevity but increased risk of major depression. The N363S polymorphism increased receptor sensitivity and was associated with increased abdominal fat, LDL cholesterol and risk of cardiovascular disease (reviewed in Koper *et al*., 2014).

The effects of stress on immune function depend on the nature of the stressor as well as the immunological function being measured. Immune system function can be evaluated by *in vitro* measures, such as the ability of natural killer cells to destroy virus-infected cells and the proliferation of T and B cells after exposure to mitogens in culture. *In vivo* measures of immune function are antibody formation after exposure to antigens and T cell-mediated delayed hypersensitivity and graft rejection. The effect of stress on resistance to disease can vary. Social stress in chickens decreases the resistance to viral diseases such as Newcastle disease or Marek's disease but increases the resistance to bacterial infections. This may be due to the action of glucocorticoids in suppressing inflammation from bacterial infection.

The reduction in cellular immunity, and the accompanying inflammation, pain and sickness behaviour, allows the animal to respond more effectively to stressful situations in the short term. However, the chronic depression of cellular immunity by long-term stress decreases the ability of the animal to fight infection by viruses, bacteria, fungi and protozoa and increases the possibility of illness. Thus, long-term stress is undesirable and makes the animal more susceptible to disease. Long-term aversive stress can also lead to organ damage, such as cardiovascular disease and gastric ulcers. Increased release of free fatty acids can lead to the formation of fatty deposits on arterial walls and can lead to atherosclerosis and hypertension. Other changes from long-term stress include adrenal hypertrophy, haemorrhage, skeletal muscle degeneration and reduced weight of other organs, such as the spleen and thymus.

The levels of anabolic hormones, including ST and IGF-1, are decreased during disease, so supplementing with anabolic hormones might reduce the catabolic effects of disease. ST treatment of critically ill patients increases protein synthesis, stimulates the immune system and improves wound healing. Use of insulin and anabolic steroids also increases nitrogen retention. Treatment of growing cattle with ST improves the immune response to experimental challenge with bacterial LPS but not to infection with parasitic coccidia. Treatment with the anabolic steroids oestradiol and progesterone improves the response to coccidia infection. It is thus apparent that treatment with anabolic hormones can improve animal responses to disease. Further research is needed in this area.

Effects of stress on reproduction

Animals that are suffering from chronic stress do not have the same reproductive success as non-stressed

animals. Acute stressors can affect uterine health, oocyte quality, ovarian function and the developmental capacity of the conceptus; this can impair reproductive function during critical periods of the reproductive cycle, such as ovulation, early pregnancy and lactation. Stress decreases the secretion of GnRH by the hypothalamus and subsequent production of LH and FSH by the pituitary and sex steroids by the gonads (Fig. 6.22). This causes decreased libido and fertility rates, lack of implantation of the fertilized ovum and retarded growth of the embryo. Perinatal stress during sensitive periods can demasculinize males and delay puberty in females.

Glucocorticoids, ACTH and CRH, as well as vasopressin and opioids such as β-endorphin, reduce GnRH secretion. IL-1 also inhibits the hypothalamic–pituitary–gonadal axis. Glucocorticoids may also directly interfere with gonadotrophin secretion by the pituitary, inhibit the production of gonadal steroids and reduce the sensitivity of target tissues to sex steroids. The initial rapid release of LH is mediated via arachidonic acid and its metabolites, while the prolonged release of LH is regulated by protein kinase C-dependent mechanisms. Glucocorticoids reduce the release of LH by inhibiting the hydrolysis of phospholipids to release arachidonic acid. Glucocorticoids also affect the feedback of gonadal steroids on the pituitary gonadotropes. In rats, glucocorticoids prevent oestrogen from sensitizing the pituitary to GnRH and thus prevent the preovulatory release of LH.

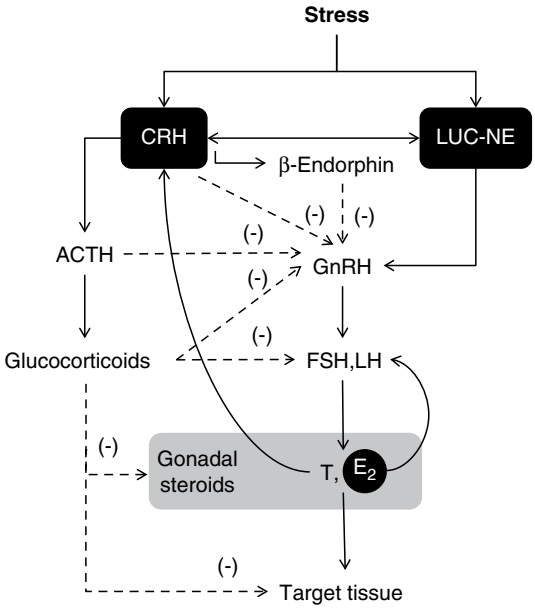


Fig. 6.22. Effects of stress hormones on gonadal function (adapted from Charmandari *et al*., 2005).

Glucocorticoids interact with reproductive hormones during early development of the neuroendocrine system. Elevated corticosteroid levels negatively affect pregnancy rates in gilts and the rate of sexual development of boars. Sexual behaviour depends on the production of gonadal hormones and may be suppressed when a stressor interferes. Sexual activity of low-ranking males is depressed when dominant animals are present, due to low production of testosterone. The stress of low social rank in females can interfere with LH production and prevent ovulation and oestrous behaviour. Paradoxically, acute stress, such as mixing with unfamiliar gilts, can increase LH production and is used for oestrus synchronization and induction of early puberty. The first few days of early pregnancy until implantation are thought to be a particularly stress-sensitive period.

Stress also negatively affects lactation. Catecholamines and opioids produced during stress reduce oxytocin production, which negatively affects milk ejection and yield.

Nutritional stress delays the onset of puberty, interferes with normal cycling in females and results in hypogonadism and infertility in males. Leptin plays a role in this effect and adequate levels of leptin are required for gonadotrophin secretion (see Section 3.5). Heat stress also inhibits gonadotrophin secretion, due to decreased production of GnRH and decreased sensitivity of the pituitary to GnRH stimulation. Long-term exposure to cold temperature also reduces LH and FSH secretion. This may be due, in part, to inadequate nutrition and lack of leptin signalling, because of the increased metabolic demand at cold temperatures. For more information, see Lucy (2019).

The effects of stress and glucocorticoids are not always negative. Glucocorticoids prepare the fetus for birth by stimulating the production of lung surfactants and the maturation of the gut. Parturition is initiated by a surge of cortisol from the fetus, which is stimulated by the production of CRH from the placenta (see Section 5.1). Infusion of the CRH receptor antagonist antalarmin into the fetus has been shown to delay parturition in sheep.

Effects of stress on growth performance

Chronic treatment of grower pigs with glucocorticoids decreases growth rate. Glucocorticoids are

catabolic and adversely affect growth performance by increasing gluconeogenesis and decreasing protein incorporation into tissues. The overall body response to stress results in decreased feed intake and growth rate, decreased efficiency of nutrient utilization for growth and increased energy requirements for maintenance (Fig. 6.23). In young animals, a loss of growth efficiency from stress can be offset somewhat by increased efficiency of nutrient use for thermogenesis. In addition to endocrine responses, stress reduces nutrient availability by decreasing appetite, gut motility and nutrient absorption and affecting the activity levels of the animal. Stress alters the gut microflora, reduces intestinal digestive enzymes and transporters and damages intestinal structural integrity. Stress also directs nutrient use towards fever thermogenesis and to support the immune system and away from growth, resulting in poorer feed conversion efficiency. While poor growth and reproduction probably indicate that the animal is in a poor state of well-being, the reverse is not always true. One cannot assume that if an animal appears to perform well, it is not under some form of distress.

Stress effects on the metabolism of different tissues depend on the responsiveness of the cells to the different hormones produced during stress. ST and IGF-1 produce anabolic effects and these hormones are generally inhibited during disease (Fig. 6.24). In addition to stimulating release of ACTH, CRH also increases the release of somatostatin (SS), which inhibits secretion of ST. ACTH and the glucocorticoids produced during stress cause catabolic effects and inhibit the effects of IGF-1 and growth factors on target tissues. In addition to their direct catabolic effects, glucocorticoids also antagonize the anabolic effects of ST and sex steroids on muscle and bone growth and their catabolic effects on adipose tissue. Thyroid hormone regulates the basal metabolic rate and affects nutrient uptake by cells. Increases in CRH due to activation of the HPA axis decrease levels of TRH and TSH. The increased level of glucocorticoids inhibits deiodinase 2 (DIO2) that converts T_4 to T_3 in peripheral tissues. In addition, changes in blood flow to different tissues affect nutrient availability and the exposure of the tissues to hormones. Blood flow changes from vasoconstriction and vasodilation are caused by arachidonic acid metabolites (prostaglandins, prostacyclins and thromboxanes) and NO.

Catabolism of glycogen in liver and muscle provides glucose, resulting in transient hyperglycaemia and then hypoglycaemia as the glycogen is depleted. Catabolism of adipose tissue provides fatty acids for energy production. Muscle protein catabolism due to TNF-α and IL-1 provides amino acids that are required by immune cells, such as glutamine. There is a priority of nutrient use among different tissues, so different body structures are differentially affected by stress. For example, the locomotor and

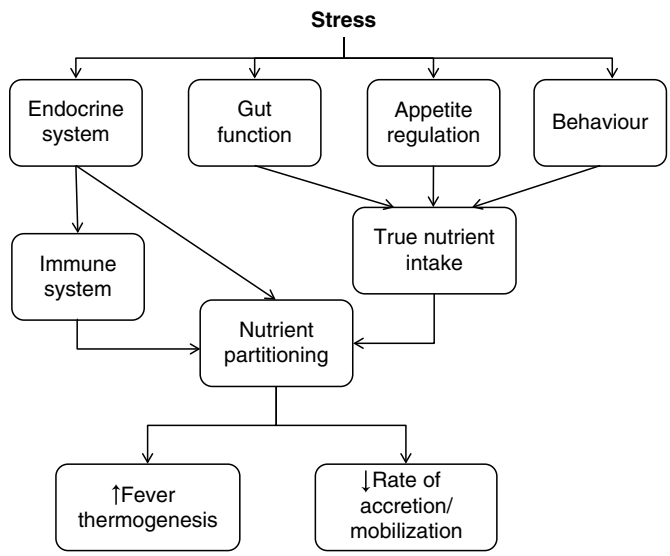


Fig. 6.23. Effects of stress on nutrient intake and utilization.

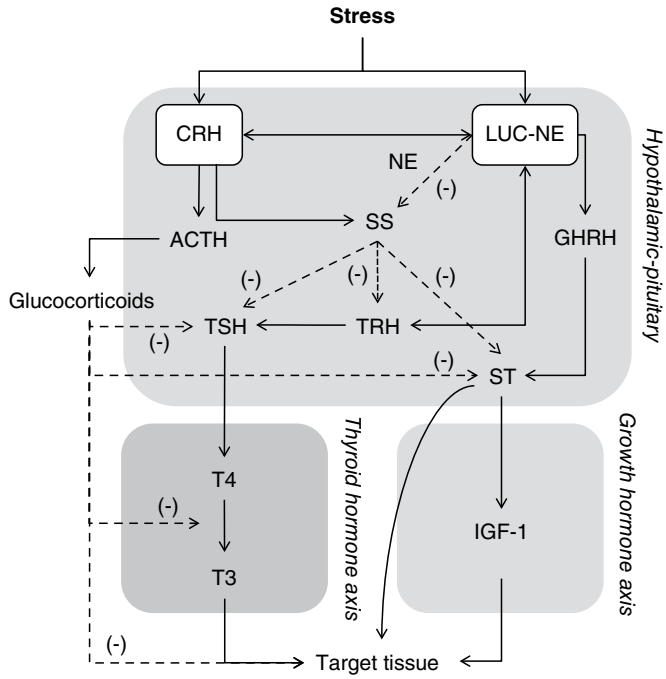


Fig. 6.24. The effects of stress on the thyroid hormone and growth hormone axes (adapted from Charmandari *et al*., 2005).

fast-twitch rectus femoris muscle is relatively unaffected, but psoas major, which is a postural muscle with slow-twitch fibres, is catabolized in cattle during stress. Severe stress results in the overproduction of stress hormones and cytokines, causing dramatic changes in metabolism, leading to catabolism, tissue wasting and pathological conditions. IL-1 induces fever and reduces feed intake. Blocking the response to the cytokines TNFα and IL-1 using receptor antagonists decreases the severity of weight loss and muscle wasting due to disease stress.

Exposure to disease organisms stimulates the immune system response and diverts nutrients away from growth. The stress of infections also reduces appetite. This may be due, in part, to the action of urocortin (see above). Intravenous injection of urocortin has been shown to delay gastric emptying and reduce food intake in rodents and marsupials. These effects are blocked by corticotrophin-releasing hormone receptor 2 (CRHR2) antagonists but not by selective antagonists for CRHR1. Glucocorticoids and proinflammatory cytokines also increase production of leptin by adipocytes to decrease appetite (see Section 3.5). Stress can also increase levels of resistin and GLP-1 and decrease ghrelin to reduce food consumption and enhance satiety.

Low levels of infection cause increased production of acute-phase proteins, reduced synthesis of muscle proteins, increased maintenance energy requirements and decreased feed intake. Sub-therapeutic levels of antibiotics are sometimes included in animal feed to reduce the activation of the stress response by subclinical infections with bacteria (see Section 3.11). Alternatively, the health status in a herd can be improved by increasing sanitation and limiting public access. For pigs, the effects of disease can be minimized with specific-pathogen-free (SPF) herds or selecting for animals with a high immune response. For further information on the effects of stress on performance, see Niu *et al*. (2022).

Summary

Stress occurs when the homeostasis of an animal is threatened and this can have dramatic negative effects on the immune system, reproduction and the growth and performance of animals. These responses are due to the production of stress hormones, including

the HPA axis and the SAM and SN systems (CRH, ACTH, glucocorticoids and catecholamines) and cytokines, as well as decreases in anabolic hormones (IGF-1, ST, thyroid hormones and sex steroids). These hormonal changes, as well as behavioural and physiological changes, can be used to assess the degree of stress that an animal is experiencing. Altering these hormonal responses can potentially reduce the effects of stress. In particular, the use of specific CRH receptor antagonists shows promise to alleviate some of the negative aspects of stress without adversely affecting other endocrine functions. Further research in this area, leading to a more complete understanding of the endocrine and metabolic responses to stress, may lead to additional treatment strategies. For more information on responses to stress at different levels of biological organization, see the review by Kassahn *et al*. (2009).

6.4 Endocrine Applications in Toxicology

Key concepts

- Endocrine disruptor chemicals (EDCs) alter the normal endocrine systems of animals to affect reproduction and metabolism via effects on nuclear receptors ER, AR, TR, AhR and PPAR.
- EDCs can act as hormone receptor agonists or antagonists, or affect hormone metabolism and transport.
- EDCs can affect hormone synthesis and catabolism by affecting the activity of CYP enzymes and dehydrogenases.
- Several different testing protocols with different *in vitro* and *in vivo* end points are available to determine the effects of EDCs.
- EDCs can be natural plant-derived compounds, such as phyto-oestrogens, or synthetic xenochemicals, such as pesticides and plasticizers.
- Both negative effects on reproduction and positive effects on lipid and glucose metabolism have been ascribed to phyto-oestrogens.
- The detrimental effects of several xenochemicals, such as DES, DDT, PCBs, BpA and TBT, have been well documented.
- Specific effects of EDCs on thyroid, adrenal and CNS function have been described.

Endocrine disruptors or modulators

The terms 'endocrine disruptor' or 'endocrine modulator' refer to exogenous chemicals that have direct endocrine effects or indirectly affect the normal endocrine systems of animals and are thus called endocrine disruptor chemicals (EDCs). The primary concern has been xenobiotic chemicals in the environment, including persistent organo-halogen compounds, such as pesticides, industrial chemicals including surfactants, and organometals. Other natural-source compounds, such as phyto-oestrogens, are also potent endocrine modulators.

Environmental pollutants have been studied for their lethal and carcinogenic effects, but many also have endocrine disruptor activities. High levels of these compounds in the Great Lakes have been linked to thyroid dysfunction, decreased fertility and hatching success, metabolic and behavioural abnormalities, altered sex ratios and compromised immune systems in various animals in this ecosystem. High levels of dioxins were linked to decreased fish populations, and to reproductive losses and mortality in mink that were fed fish from the Great Lakes. Associations between decreased sperm counts in men and the increased human exposure to synthetic chemicals over the period 1938–1998 have been claimed, but a cause-and-effect relationship has not been demonstrated conclusively. Mothers with high levels of polychlorinated biphenyls (PCBs) and dioxin had babies with statistically decreased mental development and increased incidence of learning disorders that could be detected by skilled psychologists. The development of the fetus may be more sensitive to the effects of endocrine disruptors than is an adult animal, so the age at exposure is an important consideration. There may also be a substantial period of latency between the time of exposure and the effects on the animal. The effects may be seen in subsequent generations, due to epigenetic effects from modification of DNA methylation and histone acetylation.

Natural plant-derived compounds, such as phyto-oestrogens, can also have endocrine-modulating effects that are at least as potent as those of artificial xenobiotic chemicals. Many plant and herbal extracts have been used as health aids for some time and their use is generally unregulated. Most natural compounds that affect endocrine function are biodegradable but exposure to these compounds through the diet can be high.

Since it is impossible to perform chemical analysis for all potential endocrine disruptors, certain 'indicator species', such as rats, have been used as bioindicators to assess the impact of endocrine-modulating chemicals in the environment. While this bioassay

approach measures actual effects of complex mixtures of chemicals on biological systems, the results obtained with one species have often been extrapolated to other, unrelated species. However, there are dramatic differences between species in the activities of different hormones and receptors, the ability to detoxify contaminants and in other factors that control attributes such as sex ratios. Very little is known about the comparative endocrinology of many species and the effects of a potential endocrine disruptor seen in one species may not be applicable to other species.

For more information on endocrine disruptors, see the Endocrine Society's second Scientific Statement on endocrine-disrupting chemicals (Gore *et al.*, 2015) and the World Health Organization (WHO) revised assessment of the state of the science of endocrine disruptors (Bergman *et al.*, 2012). The review by Schug *et al.* (2016) describes how the field of endocrine disruptors has developed.

Assessment of endocrine disruptor activity

The effects of EDCs on hormonal signalling can occur at several different levels. Some effects are mediated directly at the receptors; this occurs via the nuclear oestrogen receptor (ER) androgen receptor (AR), thyroid hormone receptor (TR), 9-*cis* retinoic acid receptor (RXR), aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor (PPAR), oestrogen-related receptor gamma (ERRγ) and the glucocorticoid receptor (GR) as well as cell surface receptors, by binding to the receptors to act as hormone agonists or antagonists. EDCs can also alter the expression or internalization of the hormone receptors and alter signal transduction in hormone-responsive cells. They can also interfere with signalling in the hypothalamus–pituitary–endocrine gland system and activate other signalling pathways, such as the PPAR- and AhR-mediated alterations in metabolism. EDCs can also alter the synthesis, metabolism and clearance of hormones, the transport of hormones across cell membranes and the distribution or circulating levels of hormones. EDCs can also induce epigenetic modifications and alter the fate of hormone-producing or hormone-responsive cells. Researchers have used several different assays to demonstrate the effects of potential EDCs, which are designed to investigate one or more of these potential mechanisms. For more information, see the conceptual statement by La Merrill *et al.* (2020).

In vitro assays for estimating oestrogenic activity have been developed. In one example, a cell line is transfected with a recombinant oestrogen receptor and a reporter gene (*lac-z* for β-galactosidase, *luc* for luciferase or *gfp* for green fluorescent protein) linked to an oestrogen-responsive element. Treatment with compounds that activate the oestrogen receptor increases the expression of the reporter gene (see Fig. 1.53). Competitive binding assays (see Section 2.2) for the oestrogen receptor, androgen receptor, progesterone receptor and thyroid hormone receptor have also been developed but these assays do not determine whether inhibition or activation of the receptor is occurring. Oestrogenic compounds can also stimulate cell division and expression of the oestrogen-responsive genes *c-myc*, *pS2* and the progestin receptor in oestrogen-responsive cells. The human breast tumour cell lines MCF-7 and T-47D and the ovarian cancer cell line Bg-1 have been used for this purpose. Oestrogenic compounds can also stimulate synthesis of sex hormone-binding globulin in human hepatoma cell line HepG2. Oestrogen receptor antagonists, such as ICI 182,780 and ICI 164,384, can be used to block the oestrogenic effects of the test compounds and demonstrate that specific binding to the oestrogen receptor is occurring.

In vivo bioassays for oestrogenic activity include determining the effect of treatment with the test compound on immature female rats. Oestrogenic compounds cause premature vaginal opening and increases in ovarian and uterine weight. The induction of oestrogen-responsive progesterone receptor and peroxidase genes, overall DNA synthesis in the uterus and histological examination to measure thickening and mitotic activity of the vaginal epithelium can be compared with an oestradiol-positive control.

Oestrogen induces the production of vitellogenin in the liver of juveniles or males of oviparous species, although only females normally produce vitellogenin. Test compounds can be administered *in vivo* by intraperitoneal injections or added to *in vitro* cultures of fish hepatocytes. Other effects of endocrine disruptors in fish are reduced gonad size, fecundity, egg size, hatching success and expression of secondary sex characteristics, and increased time to sexual maturity due to decreased plasma levels of sex hormones. The effects on plasma hormone levels and sperm quality and quantity can be measured in males. The Hershberger assay uses castrated male rats to identify chemicals that interact with the androgen receptor and cause proliferation of androgen-dependent tissues. The induction of specific behavioural

responses (e.g. lordosis in female rats and mounting behaviour in males) can also be measured, keeping in mind that behaviours vary among different species. Thyroid active substances can be detected using a frog metamorphosis assay with *Xenopus laevis*. Measurement of tail resorption and mortality, as well as thyroid receptor mRNA and tissue levels of T_3 and T_4, can be made.

Public concern about the effects of endocrine disruptors on wildlife and human populations has led to the development of government testing programmes. Since the late 1990s, the US Environmental Protection Agency (EPA) and the European Organization for Economic Cooperation and Development (OECD) have been developing standardized testing procedures for endocrine disruptors. These provide standardized protocols that produce reproducible results that can be compared across many different laboratories. Several different testing protocols with different end points are used (Fig. 6.25) to determine a relationship between a physiological response mediated via a hormone system and an adverse effect measured at the organism or population level. The OECD has developed a Conceptual Framework which organizes these tests into levels (1–5) of increasing biological complexity to aid in the interpretation of results by describing the Adverse Outcome Pathway for a toxin. The US EPA has organized testing in a similar manner and proposes that Tier 1 tests would first be done to screen substances for potential effects on the oestrogen, androgen or thyroid hormonal systems. If these initial screening tests were positive, then additional Tier 2 *in vivo* multigenerational testing would be done to determine the long-term effects on the organism and the dose required to obtain these effects.

Tier 1 screening tests for EDCs

Tier 1 tests include *in vitro* screens to determine if the test compound has the potential to interact with the oestrogen receptor or androgen receptor, inhibit aromatase activity or affect steroid hormone production by cell lines. This does not confirm toxicity but allows for priority setting for further testing and determination of the potential mechanism of action of the chemical. The next level of testing is short-term *in vivo* assays to determine the effects on different development stages of the animal model. This includes the effect on the development of male and female sex organs and tests in prepubertal animals, to determine effects on thyroid activity, as well as effects on neonatal animals from *in utero* exposure. Finally, in order to fully assess the potential risk of endocrine disruptors, multigenerational Tier 2 *in vivo* studies are done to assess the effects on reproduction and development. Differences among species, dose–response effects that cover the potential range of exposure, persistence in the environment, route of exposure

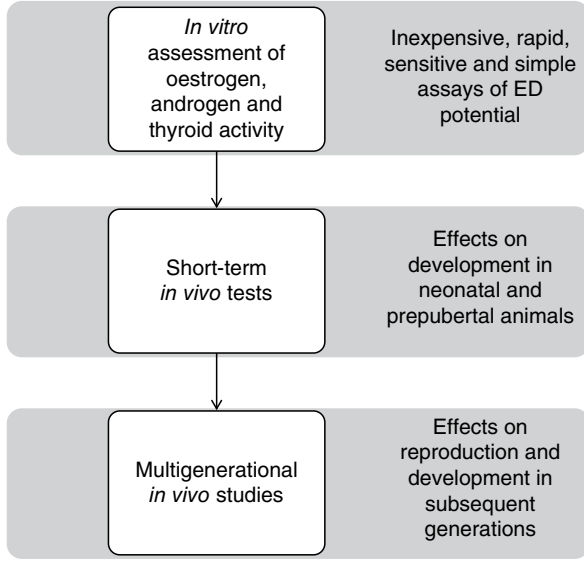


Fig. 6.25. Components of testing for EDCs.

and potential for exposure during critical periods of development need to be considered when assessing whether a chemical causes an adverse effect in the species of interest and estimating the critical effective dose of the chemical. In addition to tests with mammals (rodents), tests using various wildlife species (fish, birds, reptiles, amphibians and invertebrates) are available. Testing procedures and the endocrine end point being measured need to be carefully defined and appropriate reference chemicals that produce defined positive or negative effects are used to standardize the procedures.

Tier 1 *in vitro* tests that provide data about selected endocrine mechanisms and are endorsed by the US EPA and part of OECD level 2 include the following.

1. Oestrogen receptor (ER) binding – rat uterine cytosol. Uteri are removed from 85–100-day-old rats 7–10 days after ovariectomy, homogenized and cytosol prepared by centrifugation. A competitive binding assay and saturation binding with Scatchard analysis for the oestrogen receptor are performed using the test compound (see Section 2.2).
2. Oestrogen receptor – (hERα) transcriptional activation – human cell line (HeLa-9903). This cell line expresses the hERα and a firefly luciferase reporter construct with vitellogenin oestrogen-responsive elements (EREs) and is used to measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression.
3. Androgen receptor (AR) binding – rat prostate cytosol. Prostates are removed from 90-day-old rats 24 h after castration, homogenized and cytosol prepared by centrifugation. A competitive binding assay and saturation binding with Scatchard analysis for the androgen receptor are performed using the test compound (see Section 2.2).
4. Steroidogenesis – human cell line (H295R). Cells are incubated with test chemicals and the potential to interfere with oestrogen and/or androgen production and cell viability is measured.
5. Aromatase – human recombinant microsomes. The effect of different concentrations of the test chemical on inhibition of aromatase activity in human microsomes is measured.

Other validated *in vitro* assays such as retinoid receptor transactivation assays, androgen receptor transactivation assays and thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding) are also available (OECD, 2018).

Tier 1 *in vivo* tests that provide data about selected endocrine mechanisms and are endorsed by the US EPA and part of OECD levels 3 and 4 include the following.

1. Uterotrophic assay. Immature female or ovariectomized adult rats are exposed to test substances by subcutaneous injection for 3 days, and the effect on uterine weight is measured as an indicator of oestrogenic effects.
2. Hershberger assay. Castrated peripubertal rats are exposed to potential androgen agonists, androgen antagonists and 5α-reductase inhibitors, and the weights of androgen-responsive tissues (ventral prostate, seminal vesicle, levator ani-bulbocavernosus muscle, Cowper's glands and the glans penis) are determined.
3. Pubertal female with thyroid assay. Juvenile female rats are exposed to test chemicals by oral gavage for 20 days and effects on growth, sexual maturation and oestrous cycling, organ weights and histology, blood chemistry and levels of TSH and T_4 are determined.
4. Pubertal male with thyroid assay. Juvenile male rats are exposed to test chemicals by oral gavage for 30 days and effects on growth, sexual maturation, organ weights and histology, blood chemistry and blood levels of testosterone, TSH and T_4 are determined.
5. Amphibian metamorphosis. *Xenopus laevis* tadpoles are exposed to three different concentrations of a test chemical. Effects on mortality, development stage, hind limb length, snout–vent length, body weight and thyroid gland histology are measured at 7 days and 21 days of exposure.
6. Fish short-term reproduction assay. Mature male and female fathead minnows are exposed to the test chemical for 21 days, during which survival, reproductive behaviour, secondary sexual characteristics, fecundity and fertility are monitored. At the end of the exposure, male secondary sex characteristics, gonadal histopathology, gonado-somatic index and plasma concentrations of vitellogenin and sex steroids are determined.

Other levels 3 and 4 *in vivo* assays have been established (OECD, 2018), including the androgenized female stickleback screening assay for measuring anti-androgen activity. In this assay, fish are simultaneously treated with a model androgen (DHT) and a range of concentrations of the putative anti-androgen. Any anti-androgenic activity is detected by decreased expression of the androgen-specific

protein spiggin in the kidney. Other assays include the short-term juvenile hormone activity screening assay using *Daphnia magna*.

Tier 2 multigenerational tests

The Tier 2 multigenerational tests (OECD level 5) are designed to encompass critical life stages and processes as well as a broad range of doses that are given by a relevant route of exposure. These tests will give the long-term biological consequences of chemical exposure and identify the dose or exposure that caused the adverse effects. The effects associated with endocrine disruption may not appear until later in life or until the reproductive period is reached. Therefore, Tier 2 tests can encompass two generations and include effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life stage to sexual maturity. The tests approved by the US EPA include the following.

1. Avian two-generation toxicity test in Japanese quail. Japanese quail (*Coturnix japonica*) are exposed to the test chemical at four life stages: *in ovo*, juvenile, subadults and adults. The parental (F0) generation is exposed to the test chemical in the feed beginning at 4 weeks post-hatch, which exposes the F1 generation to the test chemical *in ovo* and also in the feed from hatch through termination. The F2 generation is fed an untreated diet. End points are egg production and viability, hatching success, survival of chicks to 14 days of age, genetic sex and onset of sexual maturation, body weight, male copulatory behaviour, morphology and histology of specific organs, and levels of sex hormones and thyroid hormones.

2. Medaka extended one-generation reproduction test. The effects of chemical exposure on reproduction and reproductive development in Japanese medaka (*Oryzias latipes*) are measured. Adult fish (F0 generation) are exposed to the test substance dissolved in water for 21 days and the development and reproduction is monitored in the F1 generation. End points are fertility, hatchability, survival and growth, and diagnostic biochemical (liver vitellogenin induction), histopathology (gonad, liver and kidney) effects and secondary sexual characteristics (anal fin papillae) in the context of the genetic sex of the individual fish.

3. Larval amphibian growth and development assay. *Xenopus laevis* are exposed to the test chemical for 35–45 days from the early embryo stage to metamorphosis and sexual differentiation is measured in the parental and F1 generations. This test measures effects on gonadal differentiation during larval development, development of secondary sex characteristics and gonadal maturation during the juvenile phase and functional reproduction and growth of adults. End points are mortality, clinical signs of disease and/or general toxicities, development time, growth (weight and length), liver somatic index, genetic/phenotypic sex ratios and histopathology of thyroid, gonads, reproductive ducts, kidney and liver.

Other level 5 tests validated by the OECD include the following.

1. Mammalian reproductive toxicity test. Adult male and female rats and their F1 offspring are exposed to test chemicals, and body weight, food and water consumption, clinical signs of toxicity, number and weight of pups, anogenital distance, sexual development end points, thyroid hormone levels, sperm parameters, vaginal cytology, organ weights, and gross and microscopic pathology of the F1 are determined.

An invertebrate life cycle test has also been proposed using Mysid shrimp. Effects on survival, growth rate, time to first brood, viable offspring, clutch size and sex ratio will be measured in *Americamysis bahia* over two generations.

Further details on the test protocols in the Endocrine Disruptor Screening Program (EDSP) of the US EPA can be found at https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-890-endocrine-disruptor-screening-program. For the OECD testing guidelines, see OECD (2018). The European Chemical Agency (ECHA) and European Food Safety Authority (EFSA) have published 'Guidance for the identification of endocrine disruptors in biocides and pesticides' (Andersson *et al.*, 2018).

Sources of endocrine disruptors

Particular interest has been expressed in chemicals that affect the actions of gonadal steroids and thus affect reproduction. This concern may be due, in part, to the experience with the use of diethylstilboestrol (DES). This is a synthetic oestrogen that was used to prevent miscarriages in pregnant women but it was later found to have teratogenic effects in the fetus. Many xenobiotic chemicals and plant-derived compounds have been shown to act as agonists of

steroid hormones, particularly oestrogens. These phyto-oestrogens and xeno-oestrogens are typically several orders of magnitude lower in activity than natural oestrogens. Phyto-oestrogens are the most potent source of environmental oestrogens, based on *in vitro* activity measurements. There is evidence that exposure to these oestrogenic compounds in the environment causes reproductive problems in wildlife. Concerns that they also cause decreased sperm counts in men and breast cancer in women continue to be hotly debated. Thus, there is considerable exposure to endocrine-modulating substances in feeds and water. This can affect animal production systems, as well as interfere with rodent bioassays designed to measure endocrine-modulating compounds.

Plant-derived endocrine modulators

A wide variety of oestrogenic and anti-oestrogenic compounds have been identified in plants (Fig. 6.26) and these may have evolved as a defence mechanism to affect reproduction in herbivores. The flavonoids are the most widely distributed group of phyto-oestrogens and include isoflavones in soya and clover, as well as flavone, flavanone and chalcones. Flavonoids are phenolic compounds with a basic structure consisting of 15 carbon atoms arranged in two aromatic rings connected by a short carbon bridge. These aromatic rings are also found in oestradiol and give flavonoids the ability to mimic the function of oestrogen. Coumestrol is derived from flavonoids and is one of the most potent oestrogenic compounds in plants. It is thought to

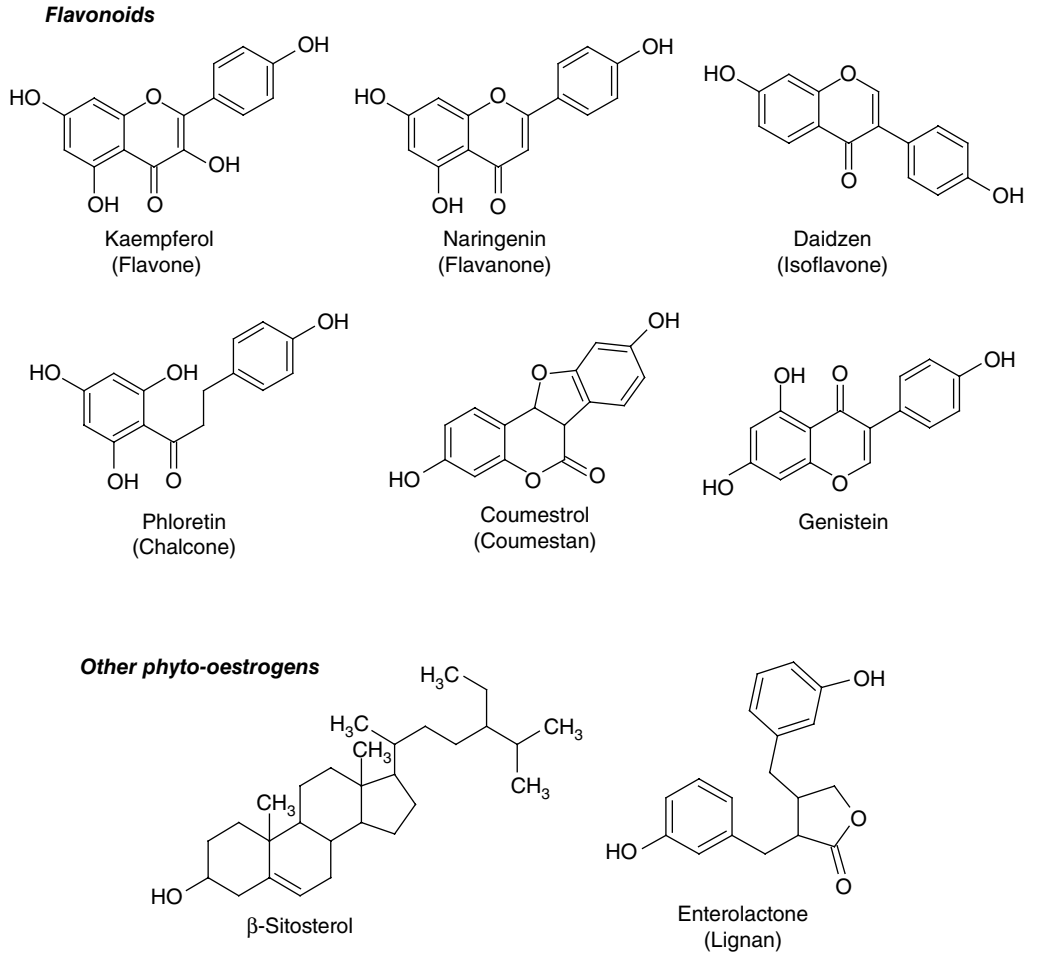


Fig. 6.26. Oestrogenic and anti-oestrogenic compounds from plants.

be responsible for 'clover disease' in sheep, which results in lesions in the reproductive organs and infertility. The isoflavonoids genistein and diadzein are present in high amounts in soya products, with 60 g of soya protein providing 45 mg of isoflavones. Genistein is four to six orders of magnitude less potent than 17β-oestradiol, but this dosage can significantly affect the menstrual cycle in women. Genistein is also a specific inhibitor of tyrosine protein kinases. Lignans such as enterolactone are present in vegetables and in high levels in flax seed. They have weak oestrogenic effects and may act as anti-oestrogens and inhibit the conversion of androstenedione to oestrone by the aromatase enzyme. Sterols such as β-sitosterol are also important phyto-oestrogens. They are present in wood and can be released from bleached kraft pulp mills at levels high enough to affect the endocrine status of fish. Bacterial degradation of plant sterols present in kraft-mill effluents can also produce androgenic steroids. The oestrogenic effects of the mycotoxin zeralenone have been known for some time as a cause of infertility and constant oestrus in pigs fed *Fusarium*-contaminated maize. Lavender and tea tree oil are essential oils that are used as alternatives for medical treatment and in personal hygiene and cleaning products, and for aromatherapy. They have oestrogenic properties and have been linked to abnormal breast growth (prepubertal gynaecomastia) in young boys.

Phyto-oestrogens can also have anti-oestrogenic effects, either by acting as oestrogen receptor antagonists or by inhibiting the synthesis of oestrogens, to modulate the endocrine system in the thyroid, liver, ovaries, bones, hypothalamic–pituitary–gonadal axis, pancreas, fat tissue and prostate. Foods and dietary supplements rich in phyto-oestrogens are commonly used as a preventive measure for breast and prostate cancer. Other health benefits such as decreased osteoporosis, cardiac disease, menopausal symptoms, obesity and metabolic disorders in lipid and glucose metabolism are also claimed from the interaction of phyto-oestrogens with oestrogen signalling, but results have been inconclusive and depend on the stage of life. For more information on phyto-oestrogens, see the reviews by Domínguez-López *et al*. (2020) and Rietjens *et al*. (2017).

Xenobiotic endocrine modulators

Xeno-oestrogens include organochlorine pesticides such as polycyclic aromatic hydrocarbons (PAHs), dioxins such as tetrachlorodibenzo-*p*-dioxin (TCDD), dichlorodiphenyltrichloroethane (DDT) and the related chemical methoxychlor, polychlorinated biphenyls (PCBs) and bisphenol A (BPA) (Fig. 6.27).

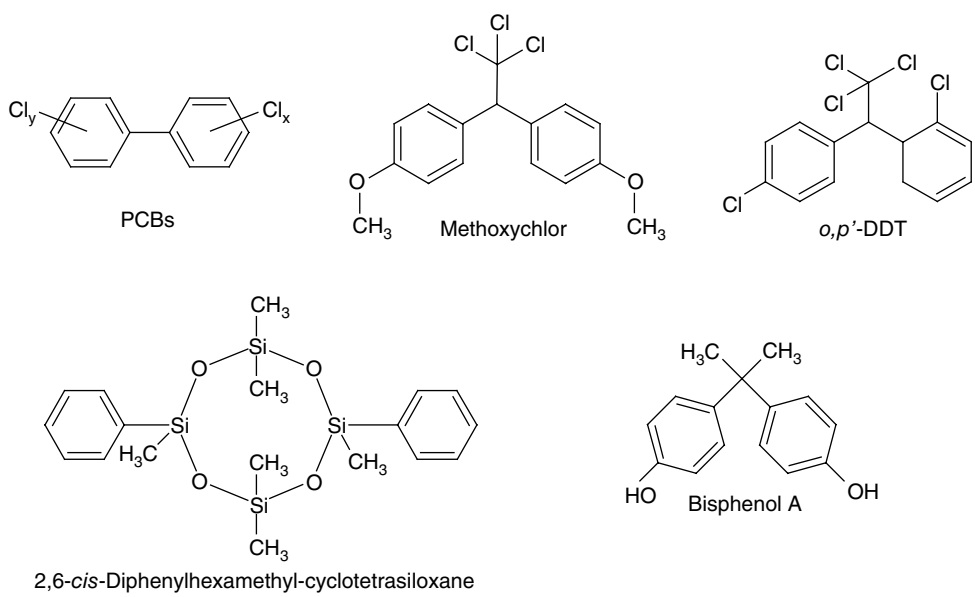


Fig. 6.27. Xenobiotic endocrine modulators.

These organochlorine pesticides are degraded very slowly and accumulate in animals at the top of the food chain to cause direct toxic effects at high levels and act as endocrine disruptors at lower levels (see below). BPA is present in polymeric form in plastics but has been found to leach out of some plastics, and several studies have proposed a relationship between exposure to BPA and the appearance of adverse health effects, such as cancer, infertility, diabetes and obesity. Regulatory agencies in many countries have determined that there is generally a very low exposure to BPA, which does not pose a health risk. However, there was a potential concern for infants, since they are more susceptible to adverse effects, and this led to banning its use in infant feeding bottles (Almeida *et al*., 2018).

Industrial chemicals such as nonylphenol, which is a breakdown product of alkoxyphenol detergents, also have oestrogenic effects. Most oestrogenic compounds are phenols or, like methoxychlor, are activated to phenols. Methoxychlor is 200,000 times less potent, nonylphenol is 300,000 less potent and PCBs are 1 million times less potent oestrogens than oestradiol. The phyto-oestrogen genistein is an order of magnitude more potent than BPA. The oestrogenic effects from organochlorines in the diet have been estimated to be 2.5×10^8 less than the oestrogen equivalents from isoflavonoids in foods. The most potent xenobiotic endocrine disruptors in mammals are the 2-phenyl-substituted cyclic tetrasiloxanes, such as 2,6-*cis*-diphenylhexamethyl cyclotetrasiloxane, which has a potency of one-tenth that of DES.

Tributyltin (TBT) was used as an anti-foulant in paints used for the hulls of ships and has dramatic effects on marine invertebrates. It results in masculinization (imposex) of females in several species of marine snails and interferes with reproduction in bivalves, including oysters and other molluscs. TBT becomes associated with nerves and ganglia and may affect the production of neurohormones. TBT affects the adipogenesis pathway by activation of nuclear receptors PPARγ and RXR. TBT also interferes with shell growth through effects on calcium channels. The resultant decline in gastropod populations has been reversed following restrictions on TBT use.

Insecticides such as juvenile hormone analogues (methoprene), which act by interfering with moulting and metamorphosis, can also interfere with the development of important marine crustaceans, including lobster, crab and shrimp.

Municipal wastewater can contain significant levels of oestrone, oestradiol and ethinyl oestradiol. These oestrogens can come from pregnant women, use of oral contraceptives and hormone replacement therapy. There may also be significant release of steroid hormones in the manure from pig production and other intensive animal-holding facilities. It has been proposed that human exposure to synthetic endocrine-disrupting chemicals is generally negligible as compared with natural compounds with higher or comparable endocrine activity (Autrup *et al*., 2020).

Indirect mechanisms of action

Effects on hormone metabolism

EDCs can interfere with hormone signalling by affecting hormone biosynthesis, transport of the hormone to the target tissue, levels of hormone binding proteins and catabolism of hormones. Xenobiotic chemicals can affect the activities of the cytochrome P450 (CYP) enzymes that are involved in the metabolism of steroid hormones. CYP enzymes in families 1–4 are responsible for the metabolism of both natural endogenous steroids and xenobiotic chemicals to more polar compounds for excretion. These families of enzymes and phase II enzymes can be induced by activation of AhR, PPAR, CAR, PXR and FXR by a variety of xenobiotic chemicals (see Section 1.3). CYP enzymes in families 11, 17, 19, 21 and 27 are involved in the synthesis of steroid hormones and these enzymes can be inhibited by xenobiotics. Thus, exposure to xenobiotic chemicals can decrease the synthesis and increase the degradation of endogenous steroid hormones, resulting in decreased levels of these hormones. Birds fed diets containing DDT, dieldrin or PCBs showed induction of hepatic CYP enzymes and increased metabolism of steroids to more polar products. Exposure of blue heron embryos to TCDD increased testosterone hydroxylase activity and 7-ethoxyresorufin-*O*-deethylase (EROD) activity (a measure of CYP1A1) measured *in vitro*. TCDD treatment did not affect plasma levels of testosterone or oestradiol *in vivo*, suggesting that the animal's endocrine system adapts to changes in steroid hormone degradation through feedback mechanisms. Ergosterol biosynthesis-inhibiting fungicides (EBIs) are widely used in agriculture and can inhibit CYP enzymes but *in vivo* treatment of partridges with a potent EBI does not affect plasma levels of steroids.

Xenobiotic chemicals can inhibit the activity of the oestrogen-specific 17β-hydroxysteroid dehydrogenase (17β-HSD) type I, which converts oestrone to the more biologically active oestradiol. A variety of flavonoid compounds inhibit 17β-HSD type I at concentrations from 0.12 to 1.2 μM. Some flavones also inhibit the 17β-HSD type II, which catalyses the 17β-oxidation of testosterone to androstenedione, and oestradiol to oestrone, producing less active steroids. Flavonoids also inhibit aromatase activity. This anti-oestrogen activity of flavonoids may explain the prevention of breast and prostate cancer attributed to diets high in phyto-oestrogens.

Organochlorine pesticides such as DDT are persistent in the environment and accumulate in animals at the top of the food chain. Fish from sites with high levels of PCBs have abnormal gonad development and decreased fecundity. Toxic effects have been shown in birds and alligators and in mammals such as seals and mink that eat these fish but it should be noted that these animals might also have high levels of other toxic compounds, such as methyl mercury. A well-known example of the effects of organochlorine pesticides is the major spill that occurred in Lake Apopka in Florida, which resulted in distorted sexual development and reproductive function in alligators (Guillette *et al*., 1994). In birds, organochlorine pesticides are thought to cause eggshell thinning, resulting in increased embryo mortality from breakage or increased permeability of the shell. This is thought to be the cause of the decline in several raptor species in North America and Europe. The major cause of this problem is thought to be *p,p′*-DDE, which is a major metabolite of *p,p′*-DDT. Commercial DDT is made up of several isomers, with 75–80% being *p,p*-DDT. The *o,p′*-DDT isomers are relatively unstable and are rarely found in the environment. These compounds can act as oestrogens themselves, as well as inducing CYP enzymes that metabolize oestrogen and thus decrease oestrogen activity. The *p,p′*-DDT isomer has also been shown to be an androgen receptor antagonist. The levels of organochlorines in the environment have decreased somewhat since the use of these chemicals was phased out in the 1970s and early 1980s and eggshell thickness in birds has increased. Levels of PCBs and TCDD are still significantly elevated in some fish in the Great Lakes.

Effects on thyroid function

Thyroid hormones are necessary for the development of neurons and they affect metabolism, growth and reproduction in all vertebrates (see Section 3.6). They are crucial in normal brain development, and mild thyroid hormone insufficiency in pregnancy is associated with impaired neurological development in the offspring. Thyroid hormones are also involved in the smoltification (conversion from a freshwater to a saltwater habitat) in salmonid fish and metamorphosis in flounder to form the asymmetrical adult. They are also required in the development of amphibians and the metamorphosis from the tadpole stage to the adult frog, which is the basis for a popular bioassay for thyroid hormone activity.

The disruption of thyroid function by EDCs can occur at several different levels (Fig 6.28) including:

- the central regulatory system in the hypothalamus and pituitary;
- thyroid hormone production at the thyroid gland by inhibition of iodine uptake by the sodium iodide symporters and the iodination of thyroglobulin by the thyroid peroxidase enzyme in the follicle cells;
- the transport of thyroid hormones by thyroid-binding globulin, thyroxine-binding prealbumin (transthyretin) and albumin;
- hormone bioavailability by inhibition of deiodinase activity and the catabolism of thyroid hormones and conjugation by the UDP-glucuronyl transferase; and
- effects on thyroid-releasing hormone receptor in the hypothalamus and pituitary, and thyroid-stimulating hormone receptor in the thyroid gland.

Cabbage and several other *Brassica* species contain potent anti-thyroid compounds. The most important of these is goitrin (vinyl-2-thioxazolidine) (Fig. 6.29), which is as active as the drug propylthiouracil, which is used for treating hyperthyroidism. Glucosinolates and cyanogenic glucosides present in foods can also be metabolized by the myrosinase enzyme present in brassica plants, as well as by intestinal bacteria, to form thiocyanate, which is goitrogenic. The soy isoflavones daidzein and genistein, as well as other flavonoids, may interfere with thyroid function by inhibiting thyroid peroxidase.

Synthetic thionamides, aromatic amines and polyhydric phenols inhibit the synthesis of thyroid hormones. There are dramatic species differences in the susceptibility of thyroid function to sulfonamides, with rats, mice, hamsters, dogs and pigs susceptible, while primates, guinea pigs and chickens are resistant. This can be explained, in part, by the inhibition of

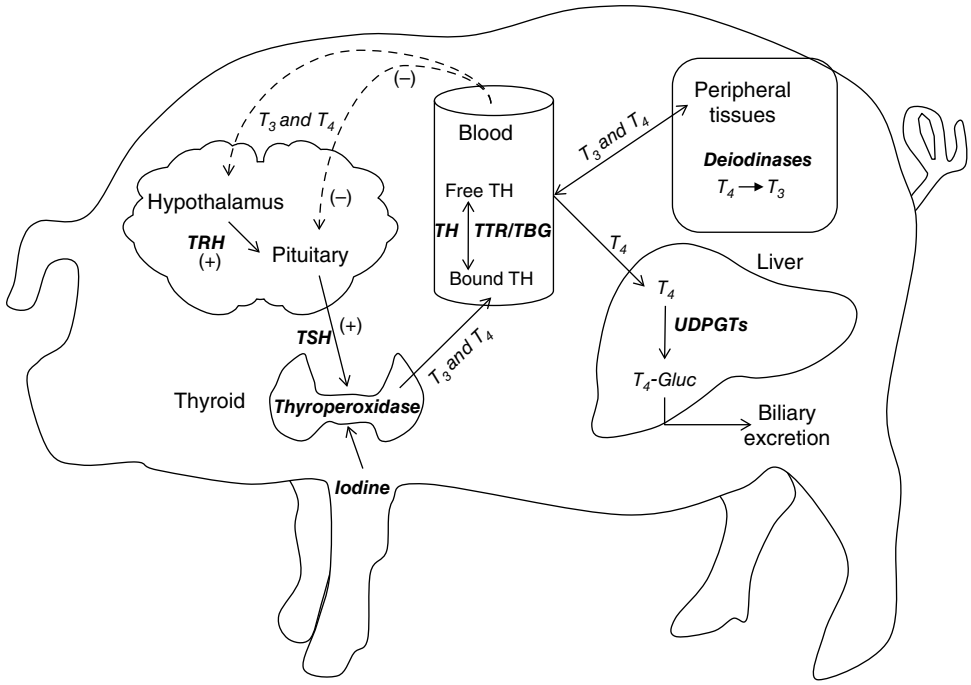
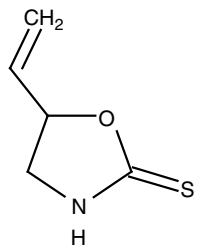


Fig. 6.28. Disruption of thyroid function by EDCs. Areas affected by EDCs are in bold italics.



Goitrin

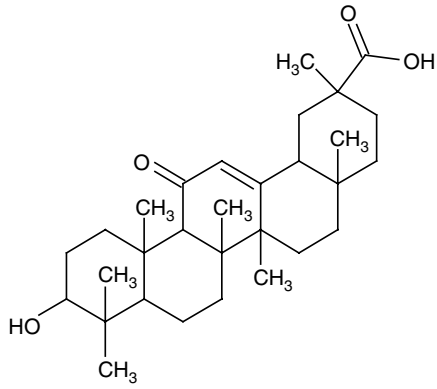
Fig. 6.29. Structure of goitrin.

thyroperoxidase by sulfamonomethoxine in the rat but not in monkeys. Thyroid activity is also higher in rats, due to high levels of TSH and the lack of thyroid-binding globulin, which increases the turnover of thyroid hormone. Sulfa drugs also induce thyroid neoplasia in the rat but are not human carcinogens. The relevance of thyroid disruption in a rodent test model to endocrine disruption in other species is thus questionable.

There is evidence that levels of thyroid hormone can be reduced in rats by exposure to high levels of PCBs, polybrominated biphenyls and dioxins. These effects are seen mainly with T_4, with no effect on active T_3, suggesting that the deiodinase activity increases to maintain T_3 levels. The decrease in T_4 is not due to decreased synthesis of T_4 but may be due to increased activity of the T_4-UDP-glucuronyl transferase or reduced binding of T_4 to the transthyretin-binding protein in the blood. These effects on thyroid hormones are not seen in birds. For more information on EDCs that affect thyroid function, see the review by Ghassabian and Trasande (2018).

Effects on adrenal function

An example of EDCs that affect adrenal function is the consumption of liquorice. Liquorice can affect the synthesis of adrenal steroids by inhibiting the 11-β-hydroxysteroid dehydrogenase enzyme type 2 and inhibiting the hepatic metabolism of aldosterone through suppression of 5-β reductase activity. This results in a mineralocorticoid effect causing retention of sodium, excretion of potassium and hypertension due to an imbalance in the renin–angiotensin–aldosterone system. Liquorice contains glycyrrhizic acid, which is 50 times sweeter than sucrose and is deconjugated to the free steroid glycyrrhetic acid (Fig. 6.30) in the intestine. Glycyrrhetic



Glycyrrhetic acid

Fig. 6.30. Structure of glycyrrhetic acid.

acid is absorbed from the intestine; it binds to mineralocorticoid receptors, potentiates the activity of aldosterone and inhibits its metabolism. A regular intake of 50 g of liquorice provides about 100 mg of glycyrrhetic acid, which is sufficient to induce clinical signs of excess mineralocorticoid activity in sensitive individuals. Glycyrrhetic acid also binds to the glucocorticoid receptor and has strong anti-inflammatory activity. For more information, see Omar *et al*. (2012).

Effects on CNS function and behaviour

Exposure to endocrine disruptors can affect the function of the CNS and alter behaviour. This can occur during early development and neonatal life, during puberty and breeding or after the behaviour is established. Hormones direct the organization of the CNS and reproductive tract during early development and are then involved in the activation of sexual dimorphic behaviours via the hypothalamic–pituitary–gonadal axis at puberty and the maintenance of behaviour during adulthood (see Section 5.1).

Testosterone, dihydrotestosterone and oestradiol are involved in male programming of the CNS and development of the reproductive tract, with the relative importance of each steroid varying among species. Treatment of neonatal female hamsters or rats with oestrogenic compounds induced male mounting behaviour when these animals were later treated with testosterone as adults. Masculinization of the CNS in this way increases the size of the sexually dimorphic nucleus in the preoptic area of the hypothalamus. Perinatal exposure to oestrogenic or anti-androgenic compounds produces developmental abnormalities in the male reproductive tract. Exposure of immature female rats to the oestrogenic pesticide methoxychlor at doses from 2 to 200 mg kg^{-1} day^{-1} induces precocious puberty within a few days of treatment. In adult rats, xeno-oestrogens reduce food consumption, weight gain and sexual behaviours.

Some pesticides act by disrupting nerve function, such as organophosphorous insecticides that act as acetylcholinesterase inhibitors and pyrethroid insecticides that affect sodium flux. These might be expected to act as endocrine disruptors, but this has not been well documented. There is evidence that endocrine disruptors impair the stress response in fish but not in birds. For more information on the effects of endocrine disruptors on the CNS, see Schug *et al*. (2015).

Summary

In summary, particular indicator species have been used for monitoring endocrine disruptors in the environment. Even though endocrine-modulating chemicals can have significant effects with *in vitro* systems, many *in vivo* studies have shown that the effects may not overwhelm the normal homeostatic control mechanisms to cause adverse effects in animals. The potential adverse effects of endocrine-modulating compounds depend on the potency, levels of exposure and time during development when exposure to the compounds occurred. Possible effects on wildlife populations are sometimes difficult to determine, due to a lack of baseline data and the presence of other environmental effects. For example, declines in fish populations can be due to habitat destruction or competition from other species. The exposure of humans in terms of oestrogenic equivalents from synthetic endocrine disruptors is estimated to be much less than that from oestrogens used for oral contraceptives or natural oestrogenic compounds in foods. However, there is mounting evidence that long-term exposure to EDCs is contributing to metabolic diseases by interfering with oestrogen, thyroid and adrenal signalling. For more information, see La Merrill *et al*. (2020).

Questions for Study and Discussion

Section 6.1 Control of broodiness in poultry

1. Describe factors that lead to broodiness in poultry.
2. How can broodiness be controlled? What long-term solutions can you suggest?

Section 6.2 Applications of pheromones

1. Compare the physiological roles of signalling and primer pheromones.
2. Describe the nature of pheromone-binding proteins (lipocalins). How do they function in the delivery and detection of pheromones?
3. How do the receptors for pheromones differ in the olfactory system and in the vomeronasal organ?
4. Describe the roles of sex pheromones in vertebrates. How do they differ among rodents, pigs, cattle, sheep and goats?
5. Describe pheromones used by fish. Comment on the additional roles of $PGF_{2\alpha}$ in fish reproduction.
6. Give examples of sex pheromones, aggregation pheromones and alarm pheromones in insects. How are pheromones detected by insects?
7. Describe the use of insect pheromones in population monitoring and in management of beneficial insects.
8. Why are chemical insecticides used more than insect pheromones in pest control?

Section 6.3 Effects of stress

1. Discuss the nature of stress. What are the behavioural, physiological and metabolic responses to stress?
2. Describe the role of the SAM, SN and HPA systems in the response to stress.
3. Describe the different roles of CRH and CRH receptors in the stress response. What are the other important roles for CRH? Describe the use of specific CRH receptor agonists and antagonists.
4. Discuss the roles of ST, thyroid hormone, DHEA and prolactin in the stress response.
5. Describe behavioural, physiological and endocrine methods to assess the level of stress. Why is it important to use more than one independent measure to assess stress?
6. Describe the systems involved in innate and acquired immunity. How does stress affect the Th1 and Th2 immune responses?
7. Describe the effects of stress on hormones in the reproductive axis.
8. Describe the overall effects of stress on nutrient utilization and growth performance. What important endocrine responses are involved?

Section 6.4 Endocrine applications in toxicology

1. Discuss the nature of endocrine disruptor chemicals. Comment on the potential modes of action of EDCs and distinguish between direct and indirect effects.
2. Outline *in vitro* and *in vivo* screening tests for endocrine disruptors and the strategy for using these tests to evaluate the effects of EDCs. Discuss potential confounding effects when extrapolating data from one species to another.
3. Give examples of plant-derived and xenobiotic endocrine disruptors.
4. What are the potential modes of action of EDCs on thyroid function?
5. Describe the potential effects of endocrine disruptors on adrenal function, CNS function and behaviour.

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Control of broodiness

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Index

APPLIED ANIMAL ENDOCRINOLOGY 3rd Edition

E. James Squires

This book explains the role of hormones in improving and monitoring the production, performance, reproduction, behaviour and health of livestock animals, focusing on cattle, pigs, sheep, horses, poultry and fish. Beginning with the principles of endocrinology and the methods to study endocrine systems, it then covers the different endocrine systems that affect different aspects of animal production and describes how these systems can be manipulated or monitored to advantage. The mechanism of action is covered, and common mechanisms and themes highlighted in order to understand potential methods for altering these systems, and stimulate ideas for the development of new methods.

A refreshed, updated resource that highlights new areas of endocrinology with applications in commercial animals, additions to this new edition include:

- information on G protein receptors, function of CREB, methods for identification of DNA regulatory sequences and DNA binding proteins, circadian rhythm and the biological clock;
- expanded coverage of *in vitro* models to include 3D cell culture and organ on a chip;
- new knowledge on gene editing, antibody production, hormone delivery methods and DNA cloning and sequencing methods;
- the role of the gut microbiome, as well as effects of antibiotics and antimicrobials;
- skin as an endocrine organ and related information on wool production and endocrine defleecing;
- updated information on protocols for assessing endocrine disruptor chemicals.

An invaluable text for students of animal science and veterinary medicine, this book also provides a useful resource for those in academia and industry interested in applications of endocrinology in animal production systems.

Cover image by Adriano, Audrey and Victoria Senatore and Kimberly Squires