The impact of diet in early life on adipose tissue growth and development in sheep

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Declaration

The work in this thesis was mainly carried out in the Division of Child Health, Obstetrics and Gynaecology at Nottingham University Hospital, Queen's Medical Centre, Nottingham, between October 2011 and October 2014. Some elements of animal work were carried out from March to May 2013 at the Sutton Bonington campus of the University of Nottingham, and I also spent three weeks in October to November 2011 assisting in animal work in Denmark under the auspices of the University of Copenhagen.

Except where otherwise indicated in the text, this thesis and the underlying work is my own work, undertaken under the supervision of Prof Michael Symonds and Prof Helen Budge of the University of Nottingham, and Prof Mette Olaf Nielsen of the University of Copenhagen. It is an accurate representation of the work performed, and no other study reproducing the work, to my knowledge, has been carried out within either the University of Nottingham or the University of Copenhagen.

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Abstract

Adipose tissue is found in two main forms: white (WAT), which stores energy; and brown (BAT), which dissipates energy as heat by means of a unique mitochondrial protein, UCP1. In large mammals, BAT is rapidly replaced by WAT after birth, but it has recently been found that functional BAT is present in human adults, which raises the possibility that it could be manipulated to burn off excess fat. The main aim of this thesis was to investigate, using sheep as a model, the effect of early nutritional interventions on fat mass and on the expression in adipose tissue of genes involved in adipogenesis, metabolism, thermogenesis and development. A secondary aim was to study their ontogeny in sternal adipose tissue.

Study A examined the effect of fat supplements given to lactating ewes on the sternal adipose tissue of their offspring. Ewes were allocated to one of three feeding groups, one control and two supplemented (sunflower or canola oil), for 28 days after parturition, and their lambs were sampled at 7 and 28 days of age. Study B investigated the effect of late gestational and postnatal diet on the sternal and subcutaneous adipose tissue of 6 month-old lambs. Twin-pregnant ewes were divided into three dietary groups for the last 6 weeks of gestation: undernourished, control or overnourished. One lamb from each twin pair was fed a control diet, and the other a high-carbohydrate, high-fat (HCHF) diet.

In the first month after birth, changes in gene expression in sternal adipose tissue were comparable to those previously described in perirenal adipose tissue, with the expression of most thermogenic genes declining to almost undetectable levels by 28 days of age. There was a disparity in the expression profiles of the two principal regulators of adipogenesis, PPARγ and C/EBPα, with expression of the former increasing with age, and that of the latter peaking at 7 days of age. A sunflower, but not canola, oil supplement fed to lactating ewes increased the relative adipose tissue weight of female, but not male, lambs at 28 days of age. Both supplements increased the plasma concentration of leptin at 7 and 28 days of age in females, but not males. Supplementation had a greater effect on gene expression at 7 than at 28 days of age, but no overall pattern emerged. Maternal undernutrition reduced birth weight in males, but not females, although body weight was unaffected by 6 months of age. A postnatal HCHF diet increased fat mass in all adipose tissue depots tested, and reduced expression of most adipogenic and metabolic genes in sternal and subcutaneous adipose tissue by around 50 %. Expression of thermogenic genes was barely detectable in either tissue at 6 months of age.

In conclusion, expression of thermogenic genes in sternal adipose tissue declines with age, a response that is unaffected by maternal fat supplementation during lactation or a sustained postnatal HCHF diet.

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List of abbreviations and acronyms

18S	18S ribosomal RNA
ADIPOQ	adiponectin
ADRB3	β_3-adrenoceptor
ALA	α-linolenic acid (18:3 *n*-3)
APS	ammonium persulfate
ARA	arachidonic acid (20:4 *n*-6)
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
AU	arbitrary unit
BAT	brown adipose tissue
BCA	bicinchoninic acid
BCS	body condition score
BEE	basal energy expenditure
BMI	body mass index
BMP	bone morphogenetic protein (eg BMP2)
BMR	basal metabolic rate
bp	base pair
BSA	bovine serum albumin
BW	body weight
CCD	charge-coupled device
cDNA	complementary DNA
C/EBP	CCAAT/enhancer-binding protein (eg C/EBPα)
CIDE	cell death-inducing DNA fragmentation factor α-like effector (eg CIDEA)
CREB	cyclic adenosine monophosphate (cAMP) response element-binding protein
CSV	comma-separated variable
C_T	threshold cycle
CV	coefficient of variation
CVD	cardiovascular disease
DAB	3,3'-diaminobenzidine
DCP	digestible crude protein
DE	digestible energy
DHA	docosahexaenoic acid (22:6 *n*-3)
DIO	diet-induced obese
DIO2	deiodinase 2
DIT	diet-induced thermogenesis
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DOHaD	developmental origins of health and disease
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
EPA	eicosapentaenoic acid (20:5 *n*-3)
ETC	electron transport chain
F	female
FABP4	fatty acid-binding protein 4
FAO	Food and Agriculture Organization (of the United Nations)
FASN	fatty acid synthase
FDG	^{18}F-fluorodeoxyglucose
FFA	free fatty acid
GC	glucocorticoid
GDM	gestational diabetes mellitus

gDNA	genomic DNA
GDP	guanidine diphosphate
GITC	guanidine isothiocyanate
GLUT4	glucose transporter 4
GOI	gene of interest
GPR120	G protein-coupled receptor 120
GR	glucocorticoid receptor
HCHF	high-carbohydrate, high-fat
H&E	haematoxylin and eosin
HFD	high-fat diet
HOXC9	homeobox C9
HPA	hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
HSL	hormone-sensitive lipase
IGF1	insulin-like growth factor 1
IHC	immunohistochemistry
IMS	industrial methylated spirit
INSR	insulin receptor (gene)
IPO8	importin 8
IR	insulin receptor (protein)
IRS	insulin receptor substrate (eg IRS1)
IUGR	intrauterine growth restriction
KDM2B	lysine (K)-specific demethylase 2B
KLF	Krüppel-like factor (eg KLF15)
KW	Kruskal-Wallis test
LA	linoleic acid (18:2 n-6)
LEP	leptin
LGA	large for gestational age
LHX8	LIM (LIN11/ISL1/MEC3) homeobox 8
M	male
ME	metabolisable energy
MHB	mitochondrial homogenisation buffer
MMLV	Moloney murine leukaemia virus
mRNA	messenger RNA
MSC	mesenchymal stem cell
mw	molecular weight
MYF5	myogenic factor 5
NCD	non-communicable disease
NFκB	nuclear factor κ (kappa) B
NR3C1	nuclear receptor 3C1 (glucocorticoid receptor)
NRTC	non-reverse transcriptase control
NST	non-shivering thermogenesis
NTC	non-template control
PCR	polymerase chain reaction
PDB	protein dissociation buffer
PGC	peroxisome proliferator-activated receptor γ (PPARγ) coactivator (eg PGC1α)
PGK1	phosphoglycerate kinase 1
PPAR	peroxisome proliferator-activated receptor (eg PPARγ)
PRDM16	PR (PRD1-BF1-RIZ1 homologous) domain containing 16
PRLR	prolactin receptor
qPCR	quantitative real-time polymerase chain reaction (PCR)
RIN	RNA integrity number
RIP140	receptor-interacting protein 140
RNA	ribonucleic acid

RNA-Seq	RNA sequencing
RPE	relative protein expression
RPLP0	ribosomal protein large P0
rRNA	ribosomal RNA
RT	reverse transcription
RT-PCR	reverse transcription (RT) polymerase chain reaction (PCR)
SDHA	succinate dehydrogenase complex A
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGA	small for gestational age
SHOX2	short stature homeobox 2
SREBF1	sterol regulatory element-binding factor 1
SREBP	sterol regulatory element-binding protein (eg SREBP1c)
SVF	stromovascular fraction (of adipose tissue)
T2DM	type 2 diabetes mellitus
T$_3$	triiodothyronine
T$_4$	thyroxine
TAE	tris-acetate-ethylenediaminetetraacetate (EDTA)
TBP	TATA (box) binding protein
TBS	tris-buffered saline
TEE	total energy expenditure
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer RNA
TTBS	tris-buffered saline (TBS) with Tween 20
TZD	thiazolidinedione
UCP	uncoupling protein (eg UCP1)
VEGFA	vascular endothelial growth factor A
v/v	volume/volume
WAT	white adipose tissue
WHO	World Health Organisation
WHR	waist over hip (circumference) ratio
w/v	weight/volume
YWHAZ	tyrosine (Y) 3- tryptophan (W) 5-monooxygenase activation (protein) zeta
ZIC1	zinc finger of the cerebellum 1

1 Introduction

This thesis explores the impact of diet in early life on the growth and development of adipose tissue in sheep. More specifically, it looks at the effect of nutrition on fat mass, and on the expression of adipogenic, metabolic, thermogenic and developmental genes in, primarily, sternal adipose tissue. It is therefore a relatively narrow and focused piece of research, which means little in isolation. The main object of this chapter, therefore, is to provide the contextual background, which at its most fundamental level is the rapidly escalating problem of human obesity. The chapter begins by defining obesity, explaining its health implications for humans, and considering its causes. It moves on to describe adipose tissue, or 'fat', the physical manifestation of obesity, discusses its various types, roles and distribution, and provides an overview of its formation and development. It then considers the effect of the early environment on adult health, before looking more specifically at the concept of the nutritional programming of obesity. It concludes by listing the advantages and disadvantages of the sheep as a model for human obesity, explaining why sternal adipose tissue was chosen as the principal tissue of investigation, stating the aims and hypotheses of the research, and summarising the characteristics and roles of the genes of interest.

1.1 Overweight and obesity

1.1.1 Definitions

Overweight and obesity are defined by the World Health Organisation (WHO) as "abnormal or excessive fat accumulation that may impair health". The standard method used by the WHO to classify overweight and obesity in humans is body mass index (BMI), which is defined as a person's weight in kilograms divided by the square of his/her height in metres ($kg\,m^{-2}$). The WHO definitions of overweight and obesity are a BMI greater than or equal to 25 or 30 respectively [WHO, 2015].

1.1.2 Health implications

Overweight and obesity can cause or exacerbate many non-communicable diseases (NCDs), either independently or in combination with other conditions, and are therefore major contributors to poor health [Kopelman, 2007]. Health risks associated with rising BMI include cardiovascular disease (CVD), stroke, type 2 diabetes mellitus (T2DM), infertility, impotency, hypertension, dyslipidaemia, sleep apnoea, osteoarthritis, gall bladder disease, musculoskeletal disorders, non-alcoholic fatty liver disease and, particularly, the metabolic syndrome [Kopelman, 2007; Visscher & Seidell, 2001].[1] The metabolic syndrome is a cluster of inter-related risk factors for CVD and T2DM which often occur together, and which may indicate an underlying pathophysiological condition [Kopelman, 2007]. Individuals with it have twice the risk of developing CVD in the following 5–10 years as those without it, and a 5-fold increase in the risk for T2DM [Alberti *et al*, 2009]. Risk factors for it include hypertension, dyslipidaemia, increased levels of fasting glucose, and central obesity, with insulin resistance as a possible linking element [Alberti *et al*, 2009]. Central (or abdominal/truncal) obesity, which is often measured in terms of waist over hip circumference ratio (WHR), may in fact be a better predictor of certain diseases than BMI. It has been suggested that fat distribution is more important in these cases than the total amount of fat [Björntorp, 1988].

1.1.3 Statistics

In recent decades, overweight and obesity have become a huge global health problem. Obesity has more than doubled since 1980, and overweight and obesity are now linked to more deaths worldwide than underweight. Globally, more than 1.9 billion adults aged 18 or over were overweight in 2014, and of these over 600 million were obese. In 2013, 42 million children worldwide under the age of five were overweight or obese [WHO, 2015]. In the USA, 78 million adults aged 20 or over (35.7 %) and 12.5 million children and adolescents (16.9 %) were obese in 2009–2010 [Ogden *et al*, 2012]. In the EU in 2008, the proportion of adults aged 18 or over who were overweight or obese was between 51.0 % and 69.3 % for men and between 37.0 % and 56.7 % for women [Eurostat, 2010]. In England, 26 % of men and 24 % of women aged 16 or over, and 16 % and 15 % respectively of boys and girls aged between 2 and 15, were obese in 2013. At the same time point, 41 % and 33 % respectively of men and women, and 14 % of both boys and girls, were overweight [HSCIC, 2013; 2015].

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[1] A small subset of obese individuals show little or no sign of cardiometabolic complications and are termed 'metabolically healthy obese' [Badoud *et al*, 2015].

1.1.4 Causes

At its simplest level, the cause of obesity can be reduced to an energy balance equation: if energy intake exceeds energy expenditure over a period of time, total body mass will increase [Hill *et al,* 2012]. At a more complex level, there are factors which influence energy intake and expenditure, and which determine the relative proportions of fat and lean body mass that contribute to any changes in total body mass.

1.1.4.1 Energy intake

Energy intake is determined by the quantity and macronutrient composition of food and drink consumed. Factors which influence food consumption include basic emotions such as hunger, appetite, satiety and mood, pathological factors such as health and disease, environmental factors such as food availability, and psychosocial factors such as food preferences, culture, peer pressure and advertising [Asp, 1999; Booth *et al,* 2001]. Variation in any of these factors can trigger changes in energy intake, and some of them have a genetic component [Rankinen & Bouchard, 2006]. In the developed world, factors that have contributed to an increase in average energy intake and the recent rise in obesity include increased affluence and the ready availability of palatable, energy-rich foods [WHO, 2015].

1.1.4.2 Energy expenditure

Total energy expenditure (TEE) is usually divided into three principal components: basal energy expenditure (BEE); diet-induced thermogenesis (DIT), also termed postprandial thermogenesis or the thermic effect of food; and physical activity [Pinheiro Volp *et al,* 2011; Weinsier *et al,* 1998; Yu *et al,* 2006]. BEE is the amount of energy expended at the basal metabolic rate (BMR), and represents energy consumed in sustaining the basic metabolic processes of cells and tissues, and in maintaining respiration, blood circulation and gastro-intestinal and renal processing [IOM, 2005]. The energy expended by DIT represents the metabolic cost of digesting, absorbing, utilising and storing nutrients after ingestion of food, plus the associated production of heat. DIT has been held to have two components: an 'obligate' component which is the energy expended in processing ingested nutrients; and a 'facultative' component which burns off some of the ingested food directly as heat, thereby reducing the amount stored as fat [Cannon & Nedergaard, 2012; Yu *et al,* 2006]. Reported figures for the three main components of energy expenditure as a percentage of TEE vary (and in any case are dependent on the activity level of any specific individual), but as a rough guide BEE makes up 50–75 %, DIT contributes a further 5–15 %, and physical activity accounts for the balance [Pinheiro Volp *et al,* 2011]. Additional energy can also be

expended on depositing new tissues for growth or pregnancy, lactation and, in unusually cold conditions, thermoregulation [IOM, 2005]. Changes in the energy expended by any of the components may be initiated by genetic, environmental or psychosocial factors [Booth *et al*, 2001]. However, physical activity is the most variable component [Hill *et al*, 2012], so its reduction as a result of increasing urbanisation and a more sedentary lifestyle in the western world is another contributory factor to the recent increase in obesity [WHO, 2015]. A further such factor is the reduction in energy expended on thermoregulation as a result of living permanently in a built, and therefore thermoneutral, environment [van Marken Lichtenbelt *et al*, 2014].

1.2 Adipose tissue and adipocytes

Adipose tissue is a specialised form of connective tissue in vertebrates which stores lipids as an energy source for the body. The principal cells of the tissue, the lipid-bearing cells, are termed adipocytes. Lipids are stored in the form of triglycerides,[2] which are esters formed from a molecule of glycerol and three molecules of one or more fatty acids. Mature adipocytes do not proliferate, and are formed from the differentiation of much smaller precursors termed preadipocytes which look similar to immature connective tissue cells [Pond, 1998]. Adipose tissue therefore grows through the proliferation and differentiation of preadipocytes (hyperplasia) or through the expansion of mature adipocytes by lipid uptake (hypertrophy). Adipose tissue also contains vascular, neuronal, immune system and connective tissue cells that, together with preadipocytes and earlier adipocyte precursors, are sometimes collectively termed the stromovascular fraction (SVF). Adipocytes and SVF cells are bound together in the tissue by a network of collagen fibres [Pond, 1998].

Historically there were thought to be two types of adipocyte, termed white and brown. In the fetus, adipose tissue (AT) may contain a mixture of both types [Symonds *et al*, 2012a], but postnatally it is found in two main forms, also termed white (WAT; Section 1.2.1, p 22) and brown (BAT; Section 1.2.2, p 23), which contain mainly white or brown adipocytes respectively. Recently, however, a distinct third type of adipocyte has been identified in WAT that has brown-like characteristics. It has been termed 'brite' (for brown-in-white) or beige, and is referred to throughout this thesis as 'brite/beige' (Section 1.2.3, p 29). All adipocyte types are innervated by the sympathetic nervous system through the release of noradrenaline, and all express the β_3-adrenoceptor [Collins *et al*, 2004; Robidoux *et al*, 2004].

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[2] Also termed triacylglycerols.

1.2.1 White adipose tissue (WAT)

1.2.1.1 Role

The principal role of WAT is to store fatty acids as a fuel reserve that can be mobilised by other cells and tissues of the body in times of energy shortage. Its secondary role is the secretion of numerous signalling molecules termed adipokines, including hormones such as leptin (Section 1.8.3.5, p 58), a marker of WAT, and adiponectin (Section 1.8.3.1, p 56), which are involved in whole body energy homeostasis [Gesta & Kahn, 2011].

1.2.1.2 Distribution

In mammals, WAT is distributed around the body in various heterogeneous 'depots', which differ in the timing and rate of adipogenesis, fat composition, metabolic function, response to hormones, gene expression, and possibly also developmental lineage [Chau *et al*, 2014; Gesta & Kahn, 2011; Gesta *et al*, 2007; Pond, 1998; Waldén *et al*, 2012; Wang *et al*, 2013]. However, WAT depots can be usefully classified into three groups: subcutaneous (between the skin and the superficial muscles); visceral (around the abdominal and thoracic organs); and intramuscular (in and around the skeletal muscles).

1.2.1.3 Adipocytes

White adipocytes are spherical in shape and resemble a signet ring in cross-section, as the bulk of their mass consists of a single large lipid droplet surrounded by a thin layer of cytoplasm that contains the nucleus, a few mitochondria and other organelles (Figure 1.1, p 23). Unlike other cells, they can expand tenfold as they take on additional fat stores [Pond, 1998], and their lipid droplet can contribute up to 85 % of their weight [Trayhurn, 2007] and over 90 % of their volume [Cinti, 2009]. Replete white adipocytes can therefore be thousands of times larger than red blood cells, immune system cells, and most brain cells [Pond, 1998].[3] Their mitochondria are thin and elongated, with randomly orientated cristae, and are generally less numerous in larger cells [Cinti, 2009]. As indicated in Section 1.2, p 21, WAT can also contain brite/beige adipocytes (Section 1.2.3, p 29).

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[3] Typical cell diameters of white adipocytes are in the range 25–200 μm [Virtanen & Nuutila, 2011].
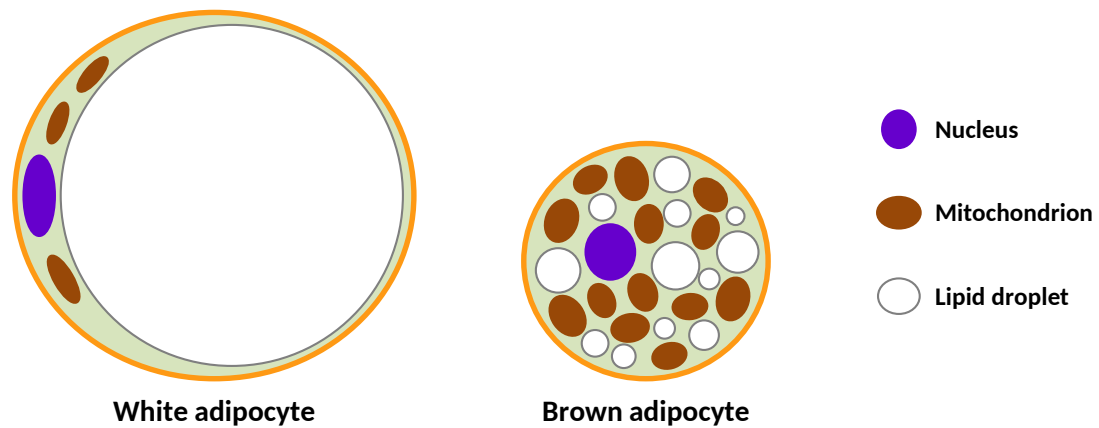
Figure 1.1 Schematic of white and brown adipocytes. White adipocytes consist of a single large lipid droplet surrounded by a thin layer of cytoplasm that contains the nucleus, a few mitochondria and other organelles. Their mitochondria are thin and elongated, with randomly orientated cristae. Brown adipocytes are much smaller than white adipocytes, they have a much higher ratio of cytoplasm to lipid, their nuclei are located closer to the centre of the cell, they are multilocular, and they have numerous specialised mitochondria, which are large, often spherical, and filled with well-structured laminar cristae. White and brown adipocytes have cell diameters in the range 25–200 μm and 15–60 μm respectively. The diagrams are not intended to be to scale.

1.2.2 Brown adipose tissue (BAT)

1.2.2.1 Introduction and principal role

Unlike WAT, BAT is unique to mammals [Cannon & Nedergaard, 2004; Pond, 1998]. It was first identified as a separate tissue as long ago as 1551 in a description of the physiology of the alpine marmot [Gesner, 1551], where it was referred to as "neither fat nor flesh".[4] It has been the subject of scientific investigation for nearly 350 years, but it is only in recent decades that its true role has been firmly established [Afzelius, 1970]. In 1961, it was demonstrated for the first time [Smith, 1961] that it is the site of non-shivering thermogenesis (NST; Section 1.2.2.2, p 24), the maintenance of an animal's body temperature in response to cold by means other than muscular movement (ie shivering). In contrast to WAT, therefore, BAT is a thermogenic organ, and its principal role is to convert stored energy into heat. It is recruited by cold acclimation and activated by cold exposure, with both processes being induced by the neuronal release of noradrenaline. If there is no further requirement for NST, BAT rapidly atrophies, often being replaced by WAT [Cannon & Nedergaard, 2004].

Small mammals can use up to 50 % of their energy to generate heat from NST in BAT at ambient temperatures [Cannon & Nedergaard, 2004], and this figure can rise to over 90 % in cold conditions [Pond, 1998]. It has been calculated that BAT can generate heat at a rate

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4 *Nec pinguitudo nee caro.*

of 350 W/kg in newborn rabbits, 381 W/kg in newborn lambs and over 500 W/kg in adult rats [Girardier, 1983], figures that are around two orders of magnitude greater than the normal metabolic rate of a mammalian tissue [Cannon & Nedergaard, 2004]. BAT therefore requires a copious supply of oxygen and nutrients, and is highly perfused with small blood vessels.

1.2.2.2 Non-shivering thermogenesis (NST)

The electron transport chain (ETC) is a mechanism by which reduced coenzymes produced during glycolysis and other catabolic processes are oxidised, simultaneously generating an electrochemical proton gradient across the inner mitochondrial membrane. This is used to power oxidative phosphorylation, a process by which the intracellular energy storage molecule adenosine triphosphate (ATP) is synthesised from adenosine diphosphate (ADP). NST functions by 'uncoupling' the ETC from oxidative phosphorylation, thereby reducing metabolic efficiency and dissipating electrochemical energy as heat (Figure 1.2, p 25). It is mediated by the unique mitochondrial protein uncoupling protein (UCP) 1 (Section 1.8.4.6, p 63), which is only expressed in brown and brite/beige adipocytes, and which has traditionally been used as the principal marker of BAT [Cannon & Nedergaard, 2004]. UCP1 activity is inhibited under normal physiological conditions, through the binding of purine nucleotides (eg ATP, guanidine diphosphate (GDP)) on its cytosolic side [Kajimura & Saito, 2014; Nicholls & Locke, 1984]. The inhibition is removed by long-chain fatty acids, possibly through partial overlapping of binding sites [Fedorenko *et al*, 2012]. Some of the signalling pathways involved in NST are shown in Figure 1.3, p 26.

1.2.2.3 Early development

There is no requirement for NST *in utero*, because the fetus is contained in a thermoneutral environment, and its highly active metabolism generates heat at about double the rate of an adult [Power et al, 1984]. Any deposition of BAT during the fetal period, therefore, is probably designed solely to prepare a newborn for the immediate cold of the extrauterine environment. The requirements for surviving in that environment are largely determined by a mammal's state of maturity at birth, being either 'precocial' or 'altricial'. Humans do not slot easily into either category,[5] but are usually classed as precocial in the context of cold-induced thermogenesis. Precocial (and human) newborns need to be able to warm themselves immediately on leaving the womb, so their thermogenic mechanisms must be fully functional by that time [Cannon *et al*, 1988]. BAT is therefore recruited prenatally and

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[5] Humans have been described as 'secondarily altricial' [Portmann, 1941].
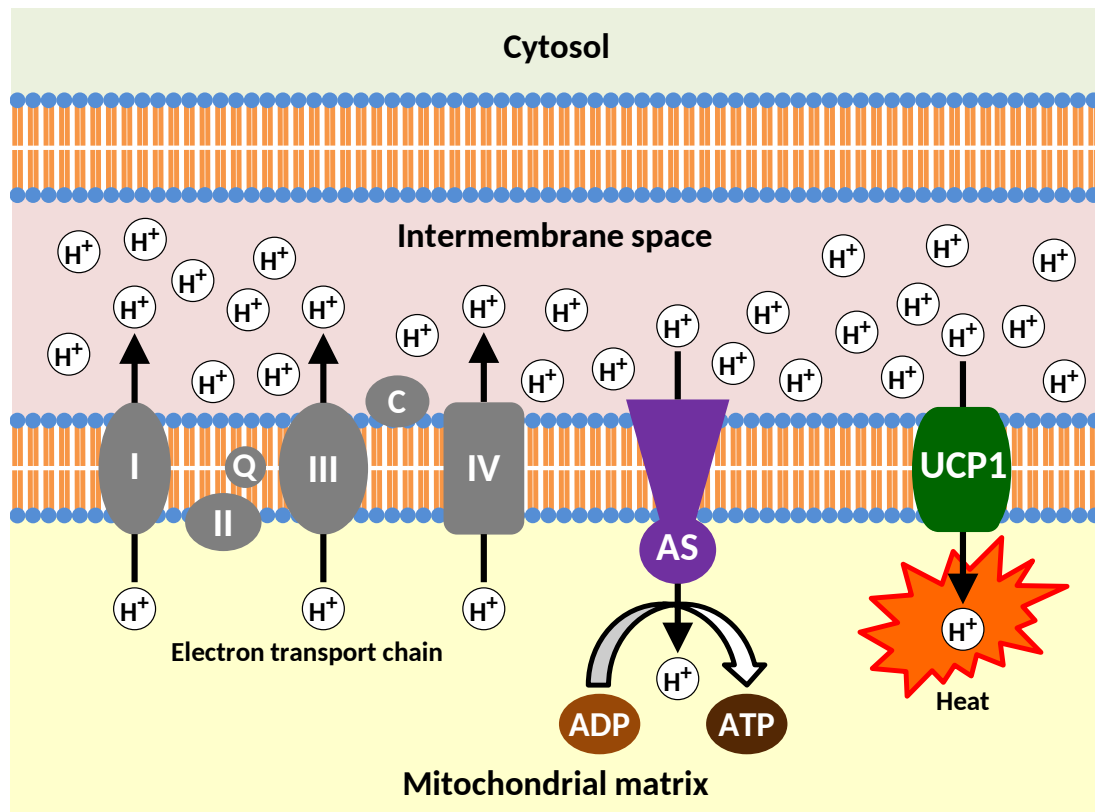
Figure 1.2 Uncoupling of the ETC from oxidative phosphorylation. The electron transport chain (ETC) is a mechanism by which reduced coenzymes produced during glycolysis and other catabolic processes are oxidised, simultaneously generating an electrochemical proton gradient across the inner mitochondrial membrane. This is used to drive oxidative phosphorylation, a process by which the intracellular energy molecule adenosine triphosphate (ATP) is synthesised from adenosine diphosphate (ADP) via the enzyme ATP synthase (AS). In non-shivering thermogenesis, however, uncoupling protein (UCP) 1, which is unique to brown and brite/beige adipocytes, dissipates the proton gradient, thereby uncoupling the ETC from oxidative phosphorylation and leading to the production of heat.

activated on birth, but soon atrophies if the newborn is not exposed to a cold extrauterine environment [Cannon & Nedergaard, 2004]. Altricial newborns rely on huddling together with their mother and siblings to keep warm, so they do not require a fully functioning thermoregulatory system until such time as they leave the nest [Cannon *et al*, 1988]. BAT is therefore recruited over the first few days after birth, before starting to decline, unless offspring are born into a thermoneutral environment, in which case it is not recruited at all [Cannon & Nedergaard, 2004]. The development of adipose tissue in general, and BAT in particular, is controlled by a variety of hormones *in utero*, and by endocrine stimulation from milk during lactation [Symonds *et al,* 2012a]. The main hormones that regulate the expression and activation of UCP1 in the fetal lamb are catecholamines, glucocorticoids (notably cortisol), triiodothyronine (T_3), prolactin and leptin [Symonds *et al, 2003;* Symonds *et al,* 2012b].
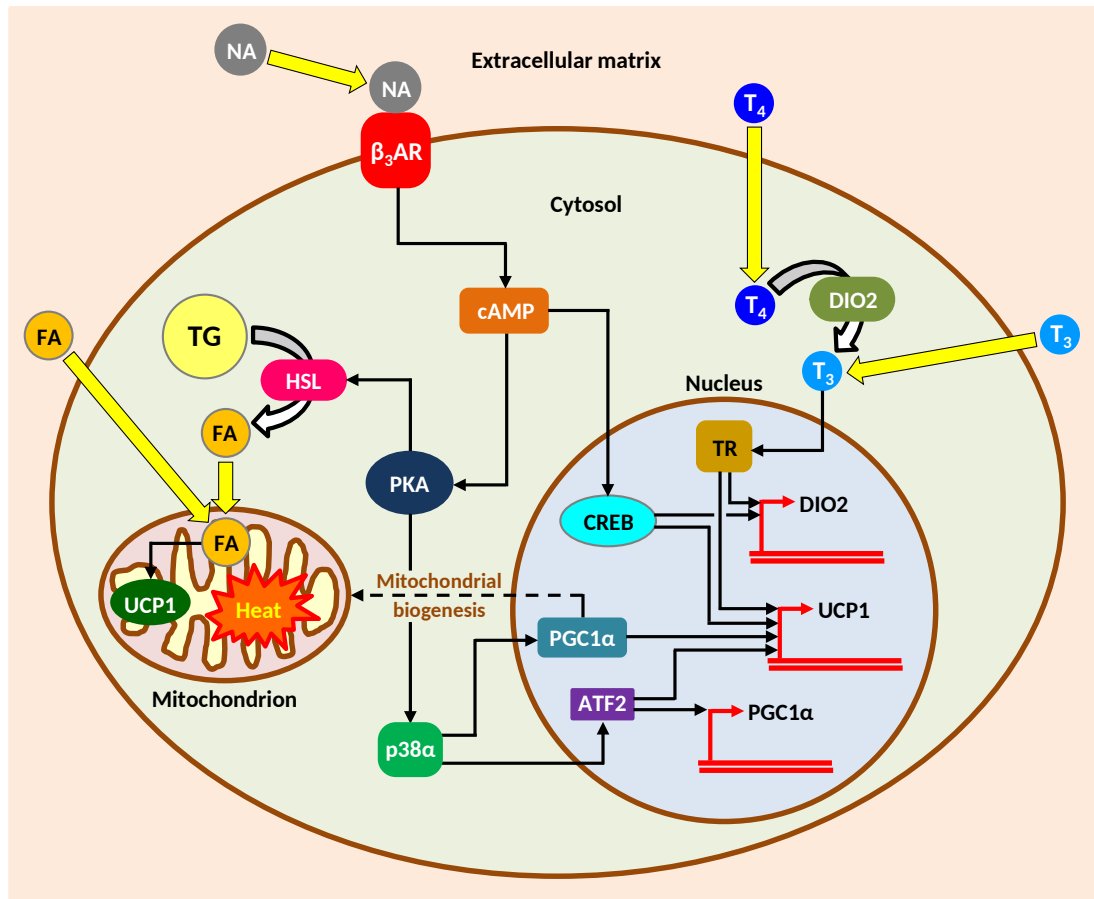
Figure 1.3 β₃-adrenergic and thyroid hormone signalling pathways involved in NST. Nor-adrenaline (NA) is released from the synapses of sympathetic neurons, binds to β₃-adrenergic receptors (β₃AR) on the cell surface of brown adipocytes, and initiates various signalling cascades that begin with the synthesis of cyclic adenosine monophosphate (cAMP). cAMP activates cAMP response element binding protein (CREB), which promotes expression of uncoupling protein (UCP) 1 and deiodinase (DIO) 2, and protein kinase (PK) A, which activates p38α mitogen-activated protein kinase (p38α) and hormone-sensitive lipase (HSL). p38α activates peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), which promotes UCP1 expression and mitochondrial biogenesis, and activating transcription factor (ATF) 2, which promotes expression of UCP1 and PGC1α. HSL mediates the breakdown of triglyceride (TG) droplets into free fatty acids (FA), which activate UCP1, and which also act as substrates for thermogenesis. UCP1 dissipates the proton gradient across the inner mitochondrial membrane, thereby generating heat. The thyroid hormone thyroxine (T₄) is converted into triiodothyronine (T₃), another thyroid hormone, by DIO2, and T₃ activates thyroid hormone receptor (TR), which promotes the expression of UCP1 and DIO2.

1.2.2.4 Other roles

In addition to its principal role in cold-induced thermogenesis, it has been suggested that BAT has a role in mediating DIT [Rothwell & Stock, 1979], more specifically the 'facultative' component of DIT that ostensibly burns off an element of food energy deliberately as heat (Section 1.1.4.2, p 20) [Yu et al, 2006]. However, although the role of BAT in cold-induced thermogenesis is now indisputable, the evolutionary case for a thermogenic mechanism to control body weight is less convincing, and some studies with mice and rats that lack the

UCP1 gene suggest that a role for BAT in DIT is unlikely [Kozak, 2010]. It may be, though, that experimental temperature is a factor in these apparently contradictory results, as UCP1-null mice become obese at thermoneutrality (29–30 °C), but not at animal house temperatures (18–22 °C) [Chechi *et al*, 2014; Feldmann *et al*, 2009].

The role of BAT does not seem to be limited to heat generation, however, and there is evidence that it has a greater involvement in glucose and lipid homeostasis [Sidossis & Kajimura, 2015; Stanford *et al*, 2013]. It may be that this is mediated through secretion of various factors (termed 'batokines' by some authors [Stanford *et al*, 2013; Townsend & Tseng, 2012]) that have autocrine, paracrine and possibly endocrine action. These include vascular endothelial growth factor (VEGF) A, insulin-like growth factor (IGF) 1, fibroblast growth factor 21, interleukin 6 and T_3 [Villarroya *et al*, 2013].

1.2.2.5 Prevalence

Until relatively recently, it was generally believed that BAT could only be found in neonates, to help them survive the cold of the extrauterine environment, in hibernators, to rapidly restore their body temperature to a normal level on arousal from hibernation, and in small mammals living in cold regions or conditions, to compensate for excessive heat loss as a result of their high surface-area to volume ratio. In large mammals, BAT rapidly atrophies after birth (and is replaced by WAT), reflecting a diminishing requirement for NST as the animal grows [Cannon & Nedergaard, 2004; Casteilla *et al*, 1989; Gemmell *et al*, 1972]. It was assumed, therefore, that BAT was lost soon after birth in humans, and that adults would possess no more than a vestigial amount. Although there was some evidence to the contrary [Huttunen *et al*, 1981; Lean, 1989; Rothwell & Stock, 1979], it was largely ignored [Nedergaard *et al*, 2007]. However, a chance observation in 1996 revitalised BAT research. In oncology, ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) is routinely used to test for tumour metastasis. During a series of such scans, a symmetrical pattern of FDG uptake was observed in the neck and thoracic paravertebral region of a number of patients [Barrington & Maisey]. As tumours are not typically symmetrical, the uptake of FDG was attributed to the contracting skeletal muscles of tense patients. The effect became well-known in the field of nuclear medicine, but even after it was suggested in 2002 that BAT might be implicated rather than skeletal muscle [Hany *et al*, 2002], it did not come to the attention of physiologists for a further five years, when Nedergaard *et al* [2007] published a review of the evidence to date. This led to a flurry of activity by BAT researchers, and a series of articles in 2009 in the New England Journal of Medicine and other publications revealed that BAT had been identified in (at least a proportion of) adult humans. Some of these studies also found that BAT is more common in women than men,

that its abundance decreases with both age and obesity, and that there is a strong seasonal variation in the amount of BAT that correlates more to photoperiod than temperature [Au-Yong *et al*, 2009; Cypess *et al*, 2009; Saito *et al*, 2009; van Marken Lichtenbelt *et al*, 2009; Virtanen *et al*, 2009; Zingaretti *et al*, 2009]. Further support for an inverse relationship between BAT and BMI in humans has since emerged [Vijgen *et al*, 2011], and it has also been confirmed that BAT in adult humans is metabolically active, and that it mediates NST [Ouellet *et al*, 2012]. It has also recently been found that a small reduction in ambient temperature, within the range of climate-controlled buildings, is sufficient to increase BAT activity in healthy human adults [Chen *et al*, 2013].

1.2.2.6 Distribution

Like WAT, BAT is dispersed around the body in heterogeneous depots [Waldén *et al*, 2012], but these tend to be fewer in number. In many species, particularly rodents, the largest depot is the interscapular [Nedergaard *et al*, 2007], whereas in the newborn sheep it is the perirenal, which makes up 80 % of all adipose tissue [Symonds *et al*, 2012a]. Small mammals generally have well-defined BAT depots, including the interscapular and retroperitoneal, throughout their lives [Giralt & Villarroya, 2013]. Various BAT depots, including the interscapular and perirenal, have also been detected in newborn humans [Aherne & Hull, 1966], though it has been reported that the interscapular depot is "quantitatively unimportant" [Lean, 1989].[6] Distribution of BAT in adult humans is similar to that of newborns, but there is no interscapular depot.[7] The two main depots are the supraclavicular and neck, with the former being the larger. Mediastinal, paravertebral, pericardial, suprarenal and para-aortic depots can also be found [Nedergaard *et al*, 2007].

1.2.2.7 Adipocytes

Brown adipocytes in BAT depots are often termed 'classical' brown to distinguish them from brown-like brite/beige adipocytes in WAT depots. They are much smaller than white adipocytes,[8] and the individual cells are much harder to distinguish in a histological tissue sample. In contrast to white adipocytes, they are polygonal in shape, they have a much higher ratio of cytoplasm to lipid, their nuclei are located closer to the centre of the cell,

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[6] It has recently been confirmed that the interscapular depot in human infants contains classical brown adipocytes [Lidell *et al*, 2013].

[7] The lack of an interscapular BAT depot may explain why the presence of functional BAT in adult humans was refuted for so long [Nedergaard *et al*, 2007].

[8] Typical cell diameters of brown adipocytes are in the range 15–60 μm [Virtanen & Nuutila, 2011].

they are multilocular (containing many small lipid droplets dispersed throughout the cytosol) and they have numerous specialised mitochondria to generate the required heat (Figure 1.1, p 23). Their mitochondria are large, often near-spherical, and filled with well-structured laminar cristae [Cinti, 2009]. It is the high mitochondrial density and profuse vascularisation that account for the distinctive colour of BAT. The different features of white and classical brown adipocytes are summarised in Table 1.1, below.

1.2.3 Brite/beige adipocytes

It has been known for some time that brown-like adipocytes are present in the WAT depots of rodents [Young et al, 1984] and that they can be further induced by chronic exposure to cold or by treatment with a β_3-adrenoreceptor agonist [Cousin et al, 1992; Guerra et al, 1998; Xue et al, 2009; Young et al, 1984] or a peroxisome proliferator-activated receptor (PPAR) γ agonist [Fukui et al, 2000; Laplante et al, 2003; Rong et al, 2007; Sell et al, 2004; Wilson-Fritch et al, 2004]. The effect is greatest in the inguinal (subcutaneous) depot and least in the epididymal (visceral) depot [Petrovic et al, 2010]. It was thought that these were classical brown adipocytes of the sort that might be found in the interscapular BAT depot, but it has recently been discovered that they are molecularly distinct. Although they express UCP1 and other core thermogenic genes, are multilocular, and contain numerous mitochondria, they lack some transcription factors associated with classical brown adipocytes, and retain expression of some genes characteristic of white adipocytes [Harms & Seale, 2013; Petrovic et al, 2010]. Unlike classical brown adipocytes, they express UCP1 at low levels in the basal state [Wu et al, 2012], but they are fully thermogenic and, when stimulated, have a similar thermogenic capacity to classical brown adipocytes [Okamatsu-Ogura et al, 2013; Shabalina et al, 2013]. They are termed 'brite' [Petrovic et al, 2010], 'beige' [Ishibashi & Seale, 2010] or

Table 1.1 Comparison of white and brown adipocytes.

Feature	White adipocytes	Brown adipocytes
Function	Energy storage	Heat production
Lipid droplets	Unilocular	Multilocular
Cell diameter	25–200 μm	15–60 μm
Cell shape	Spherical	Polygonal
Characteristic protein	Leptin	UCP1
Development	From MYF5− progenitors	From MYF5+ progenitors
Mitochondria		
Abundance	Few	Many
Morphology	Small, thin, elongated	Large, fat, near-spherical
Cristae	Randomly orientated	Well-structured, laminar

'inducible brown' [Schulz *et al*, 2011] adipocytes. It has not yet been firmly established whether they arise from undetected brite/beige precursors, from transdifferentiation of white adipocytes or their precursors [Gesta *et al*, 2007], from "masked" unilocular brown-like mature adipocytes that revert to a more typical 'brown' phenotype on stimulation [Cousin *et al*, 1992], or from a combination of some or all of these routes.

While brite/beige adipocytes have historically been treated as a subset of cells in WAT depots, Waldén *et al* [2012] have recently suggested that traditional BAT and WAT depots in mice could be reclassified as brown, brite/beige or white adipose tissue depots based on their molecular signatures. Using zinc finger of the cerebellum (ZIC) 1, homeobox (HOX) C9 (Section 1.8.5.1, p 63) and transcription factor (TCF) 21 as markers for brown, brite/beige and white adipose tissue respectively, they categorised classical BAT depots as brown, subcutaneous WAT depots as brite/beige, and most visceral WAT depots as white. More recent work, however, suggests that, in mice at least, HOXC9 is not an effective marker of brite/beige adipose tissue [de Jong *et al*, 2015].

1.2.4 Developmental pathways

It is generally believed that most brown and white adipocytes develop from mesenchymal stem cells (MSCs) originating in the mesoderm [Billon *et al*, 2008; Gesta *et al*, 2007], with a subset around the head developing from neural crest cells originating in the neurectoderm [Billon *et al*, 2008]. The complete pathways, and the number and nature of intermediaries between MSCs and preadipocytes, have not yet been fully mapped [Billon *et al*, 2008; Gesta *et al*, 2007], but it had been long thought that MSCs gave rise to a common early adipocyte precursor, termed an adipoblast, which ultimately developed into a committed brown or white preadipocyte [Gesta *et al*, 2007]. In 2007, however, it was found that that a divergence in the white and brown lineages occurs before a common adipoblast stage [Timmons *et al*, 2007], and it was later found that, unlike white and brite/beige adipocytes, classical brown adipocytes develop under the control of a transcription factor, PRD1-BF1-RIZ1 homologous domain containing (PRDM) 16 (Section 1.8.5.3, p 64), from skeletal muscle precursors that express myogenic factor (MYF) 5 (Figure 1.4, p 31) [Seale *et al*, 2008]. Recently, however, the picture has become even more complicated. It now appears that at least a subset of brite/beige (but not classical brown) adipocytes have a smooth muscle-like origin [Long *et al*, 2014], and that some white (and possibly also brite/beige) adipocytes are MYF5-positive [Sanchez-Gurmaches *et al*, 2012]. Both white and brite/beige adipocyte populations may therefore be more heterogeneous than previously imagined. Finally, there is also evidence that white adipocytes can transdifferentiate into brite/beige adipocytes on cold exposure, via an intermediate form termed a 'paucilocular' adipocyte [Barbatelli *et al*, 2010].
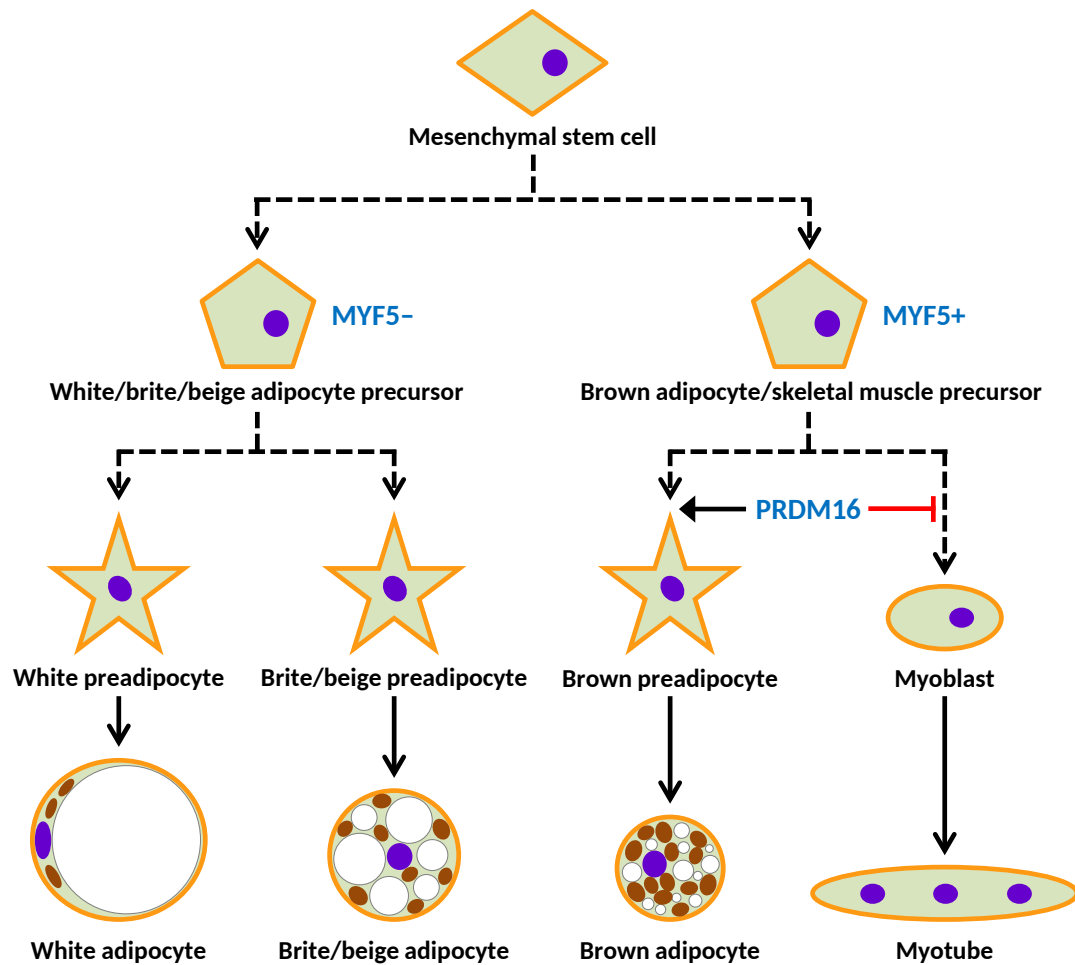
Figure 1.4 Principal developmental pathways of adipocytes. Most adipocytes develop from mesenchymal stem cells (MSCs) originating in the mesoderm. The complete pathways, and the number and nature of intermediary cells between MSCs and preadipocytes, have not yet been fully mapped [Billon *et al*, 2008; Gesta *et al*, 2007], but it is known that white and brown lineages diverge before reaching a common adipocyte precursor stage [Timmons *et al*, 2007]. Unlike white and brite/beige adipocytes, brown adipocytes develop under the control of the transcription factor PRD1-BF1-RIZ1 homologous domain containing (PRDM) 16 from skeletal muscle precursors that express myogenic factor (MYF) 5 [Seale *et al*, 2008]. However, it has recently been discovered that at least a subset of brite/beige adipocytes have a smooth muscle-like origin [Long *et al*, 2014], and that a subset of white (and possibly brite/beige) adipocytes are MYF5-positive [Sanchez-Gurmaches *et al*, 2012] (not shown).

1.2.5 Adipogenesis

Adipogenesis is the process by which precursors in the SVF of adipose tissue differentiate into mature adipocytes. It is thought to occur in two stages: (1) commitment of MSCs to a preadipocyte fate; and (2) terminal differentiation [Cristancho & Lazar, 2011]. Pluripotent MSCs provide a virtually unlimited source of adipocyte precursors, though they are also capable of developing into myocytes, chondrocytes and osteocytes. With the appropriate stimulation, they undergo a multistage programme of commitment to the adipocyte lineage, giving rise to preadipocytes [Tang & Lane, 2012]. Recruitment to the adipocyte lineage

in vivo seems to be triggered by excess energy intake and elevated glucose uptake over an extended period of time [Shepherd *et al*, 1993]. Bone morphogenetic protein (BMP) 2 and BMP4 are among the factors that promote the commitment of MSCs to preadipocytes [Tang & Lane, 2012].

The mechanism of terminal differentiation has been mainly studied using immortalised fibroblast cell lines, such as 3T3-L1 and 3T3-F442A, that can be induced to differentiate into adipocytes *in vitro* after administration of a hormonal cocktail [Rosen *et al*, 2002; Siersbæk *et al*, 2010]. Following induction, growth-arrested preadipocytes undergo several rounds of mitosis (known as mitotic clonal expansion), and a transcriptional cascade is activated that induces the expression of adipogenic genes and, ultimately, numerous genes characteristic of mature adipocytes, such as fatty acid binding protein (FABP) 4 (Section 1.8.3.2, p 56), glucose transporter (GLUT) 4, leptin (Section 1.8.3.5, p 58) and adiponectin (Section 1.8.3.1, p 56). Cells lose their fibroblastic morphology, take up triglycerides from the cytosol, and adopt the appearance of mature adipocytes [Cristancho & Lazar, 2011; Tang & Lane, 2012]. Over 40 % of the soluble protein in a mature adipocyte is accounted for by differentiation-dependent gene expression [Tontonoz *et al*, 1993].

The most important transcription factors involved in terminal differentiation include the ligand-activated nuclear receptor PPARγ (Section 1.8.2.3, p 54), the CCAAT/enhancer-binding proteins (C/EBPs), α (Section 1.8.2.1, p 53), β, and δ, and sterol regulatory element-binding protein (SREBP) 1c [Rosen *et al*, 2002]. The hormonal induction of differentiation is rapidly followed, within about four hours, by expression of C/EBPβ and C/EBPδ [Cao *et al*, 1991; Tang & Lane, 1999; Yeh *et al*, 1995]. After a delay of some 18–24 hours, C/EBPβ induces expression of PPARγ and C/EBPα, which in turn coordinate the expression of numerous genes that ultimately produce the mature adipocyte phenotype [Tang & Lane, 2012]. The importance of C/EBPβ in the process is illustrated by its ability to promote adipogenesis in the absence of hormonal induction when overexpressed in 3T3-L1 cells [Yeh *et al*, 1995].

PPARγ and C/EBPα are the principal regulators of adipogenesis, and positively regulate each other's expression [Rosen *et al*, 2002]. C/EBPα also maintains its own expression by autoactivation [Christy *et al*, 1991]. As with C/EBPβ, ectopic expression of either PPARγ or C/EBPα in 3T3-L1 cells is sufficient to induce adipogenesis [Freytag *et al*, 1994; Lin & Lane, 1994; Tontonoz *et al*, 1994]. Loss of function studies have shown that C/EBPα is clearly required for adipogenesis both *in vivo* and *in vitro*. However, C/EBPα-null fibroblasts can be induced to differentiate when PPARγ is added, albeit with reduced lipid uptake and other defects in the adipocyte phenotype, strongly suggesting that the principal role of C/EBPα in adipogenesis is the induction and maintenance of PPARγ levels [Rosen *et al*, 2002; Wu *et al*, 1999]. In contrast, while PPARγ is also clearly required for adipogenesis *in vivo* and *in*

vitro [Barak *et al*, 1999; Kubota *et al*, 1999; Rosen *et al*, 1999], the addition of C/EBPα cannot induce differentiation of PPARγ-null cells [Rosen *et al*, 2002]. PPARγ is considered, therefore, to be the 'master regulator' of adipogenesis [Farmer, 2006]. However, *in vitro*, C/EBPα is required for the development of insulin sensitivity [El-Jack *et al*, 1999; Wu *et al*, 1999], and PPARγ, C/EBPα and C/EBPβ are all required for the sustained expression of most adipocyte genes [Lefterova *et al*, 2008]. Curiously, while C/EBPα is required for the development of WAT *in vivo* in the adult, it is not required for embryonic adipogenesis [Wang *et al*, 2015] or the development of BAT [Linhart *et al*, 2001]. It is likely that other C/EBPs, notably C/EBPβ, act to maintain PPARγ expression in BAT [Farmer, 2006], as it has been shown that C/EBPβ can reprogramme 3T3-L1 cells to a brown adipocyte phenotype [Karamanlidis *et al*, 2007].

The role in adipogenesis of SREBP1c, transcribed by the sterol regulatory element-binding factor (SREBF) 1 gene (Section 1.8.2.4, p 55), is less clear. It is expressed at higher levels in committed preadipocytes than in other fibroblasts, and is further elevated during terminal differentiation [Tontonoz *et al*, 1993], with the increased expression occurring at a similar time to induction of PPARγ (ie around a day after initiation). Ectopic expression of a dominant negative SREBP1c inhibits the differentiation of 3T3-L1 preadipocytes, whereas ectopic expression of SREBP1c in NIH-3T3 cells, a relatively non-adipogenic fibroblast line, induces differentiation [Kim & Spiegelman, 1996]. SREBP1c has been shown to enhance the adipogenic effect of PPARγ *in vitro* by contributing to the production of endogenous PPARγ ligands [Kim & Spiegelman, 1996; Kim *et al*, 1998b], though various studies indicate that it may not be necessary for adipogenesis *in vivo* [White & Stephens, 2010].

Other important transcription factors involved in adipogenesis include the Krüppel-like factors (KLFs), signal transducer and activator of transcription 5, glucocorticoid receptor (GR; Section 1.8.2.2, p 54) and cyclic adenosine monophosphate response element-binding protein (CREB). A common characteristic of these factors is that they act by regulating the expression or activity of PPARγ and/or the C/EBPs, which emphasises the importance of these key regulators in the differentiation process [Siersbæk *et al*, 2010]. A diagram of terminal differentiation is shown in Figure 1.5, p 34.

Finally, the transcription factor PRDM16 (Section 1.8.5.3, p 64) is crucial for brown fat adipogenesis, and has been termed the master regulator of brown adipocytes [Lidell *et al*, 2014]. It forms a complex with C/EBPβ in myoblast precursors or brown preadipocytes to induce expression of PPARγ and PPARγ coactivator (PGC) 1α (Section 1.8.4.4, p 61). PRDM16 then coactivates PPARγ and PGC1α, which in turn induce the BAT differentiation program [Kajimura *et al*, 2009; Kajimura *et al*, 2010; Seale *et al*, 2008]. It also forms a complex with C-terminal binding proteins 1 and 2 to repress the expression of white adipocyte-specific genes [Kajimura *et al*, 2008].
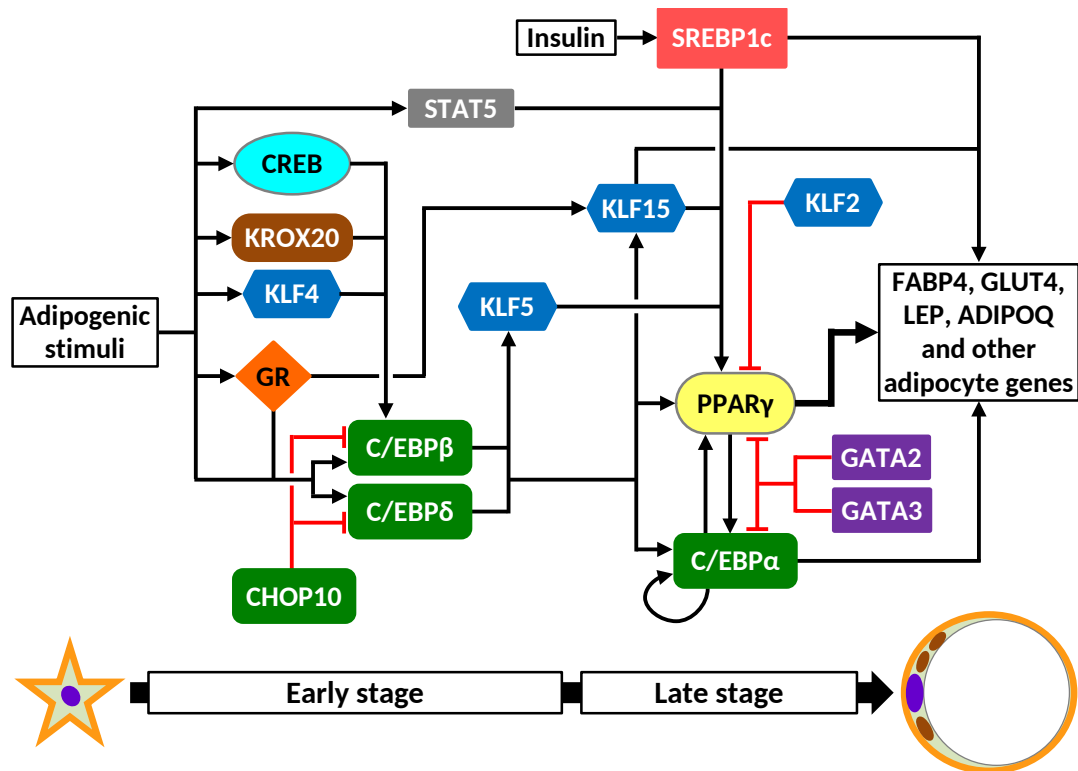
Figure 1.5 Transcriptional control of terminal adipocyte differentiation. Adipogenesis is the process by which adipocyte precursor cells differentiate into mature adipocytes, and is thought to occur in two stages: (1) commitment of mesenchymal stem cells to a preadipocyte fate (not shown); and (2) terminal differentiation from a preadipocyte to a mature adipocyte. Some of the important factors involved in controlling the latter stage are shown in the figure. The hormonal induction of differentiation is rapidly (within about 4 hours) followed by expression of CCAAT/enhancer-binding proteins (C/EBPs) β and δ (the early stage). After a further 18–24 hours, these induce the expression of peroxisome proliferator-activating receptor (PPAR) γ, the master regulator of adipogenesis, and C/EBPα, which coordinate the expression of numerous genes that ultimately produce the mature adipocyte phenotype (the late stage). Other factors that are involved in the positive regulation of adipogenesis are cyclic adenosine monophosphate response element-binding protein (CREB), zinc finger protein KROX20, Krüppel-like factors (KLFs), glucocorticoid receptor (GR), signal transducer and activator of transcription (STAT) 5 and sterol regulatory element-binding protein (SREBP) 1c. Inhibitors of adipogenesis include C/EBP homologous protein (CHOP) 10, KLF2 and GATA-binding proteins 2 and 3. Some of the important genes characteristic of mature adipocytes are fatty acid-binding protein (FABP) 4, glucose transporter (GLUT) 4, leptin (LEP) and adiponectin (ADIPOQ).

1.2.6 Clinical significance of non-shivering thermogenesis

As was explained in Sections 1.2.2.1, p 23, and 1.2.3, p 29, MYF5-positive brown adipocytes in classical BAT, and MYF5-negative brite/beige adipocytes in WAT, are thermogenic, and dissipate energy via UCP1 [Petrovic *et al*, 2010; Seale *et al*, 2008]. In animal models, genetic manipulations that have increased the amount of BAT or brite/beige adipocytes have had protective effects against obesity and T2DM. For example, ectopic expression of PRDM16 in murine subcutaneous (but not epididymal) WAT induced the development of brown-like adipocytes, increased energy expenditure, reduced weight gain and improved glucose tolerance in animals exposed to a high fat diet [Seale *et al*, 2011]. Similarly, overexpression

of cyclooxygenase (COX) 2, a downstream mediator of β-adrenergic signalling, in murine WAT induced 'BAT' recruitment, increased energy expenditure, and protected against the obesogenic effects of a high fat diet [Vegiopoulos et al, 2010].

In a human context, the discovery that functional BAT or brite/beige adipose tissue is present in adults has potential clinical implications. The activation of even small amounts of BAT or brite/beige adipose tissue could lead to new treatments for obesity, T2DM and other metabolic disorders [Gesta et al, 2007]. The use of mitochondrial uncoupling as a dietary aid in humans is not new, and has proved to be very effective. Chemical uncoupling agents such as 2,4-dinitrophenol (DNP) were used in the USA in the 1930s, both clinically and in self-medication products [Parascandola, 1974], and are still used by bodybuilders today, albeit illegally. However, they are not tissue-specific, and their uncontrolled use led to unpleasant, and sometimes fatal, side-effects [Grundlingh et al, 2011]. In contrast, the controlled recruitment and activation of BAT or brite/beige adipose tissue could increase energy expenditure, and thereby reduce obesity, without necessarily causing problems in other organs [Seale et al, 2008].

1.3 Developmental origins of health and disease

1.3.1 Introduction

Research into human NCDs has traditionally focused on genetic or adult lifestyle factors [Gluckman et al, 2010b], but over recent decades it has become increasingly clear that adult health outcomes and phenotypes can also be linked to specific events at critical windows in the early stages of human development, a phenomenon which has become known as the 'developmental origins of health and disease' (DOHaD) hypothesis [Barker, 2007; de Boo & Harding, 2006]. The first evidence of this effect arose from animal studies in the early 1960s, where it was found that undernourishment of rats at various stages of development had different long-term effects on weight gain [Widdowson & Kennedy, 1962; Widdowson & McCance, 1960; 1963]. This was followed by a number of human epidemiological studies towards the end of the last century that linked low birth weight, infant mortality and/or childhood poverty to adult CVD in the same cohorts [Barker & Osmond, 1986; Barker et al, 1989; Forsdahl, 1977; Osmond et al, 1993]. Numerous subsequent studies have linked low birth weight and/or early growth patterns to the later risk of obesity and other NCDs, such as T2DM, hypertension, glucose intolerance and insulin resistance [McMillen & Robinson, 2005; Newsome et al, 2003; Whincup et al, 2008].

The results of these studies gave rise to the idea that adult health might be influenced by the intrauterine environment [Barker, 1990; Barker & Osmond, 1987; Barker et al, 1989].

In particular, Barker and Osmond [1986] proposed that poor nutrition in early life could increase susceptibility to the harmful effects of an affluent diet. In addition to nutrition, other environmental influences in the developmental period include fetal number and sex, maternal body composition, age, parity, disease, stress, alcohol consumption, drug use and smoking [Kramer, 1987; Symonds & Budge, 2009], and external factors such as temperature and altitude [McCance & Widdowson, 1974].

The adverse effects of low birth weight and poor early nutrition on adult health were later confirmed by further animal studies. These enabled the conditions to which the fetus was exposed to be controlled, the timing of any exposure to be varied, and maternal, fetal and offspring tissues to be sampled [Warner & Ozanne, 2010]. Various animal models have been used, particularly rats [Langley & Jackson, 1994; Woodall *et al*, 1996], because of their short gestation periods and lifespans, but also large animals such as sheep [Gopalakrishnan *et al*, 2004; Hawkins *et al*, 2000], pigs [Poore *et al*, 2002; Poore & Fowden, 2003] and non-human primates [Cox *et al*, 2006]. Finally, further evidence for a non-genetic influence on human health has been provided by studies that link differences in birth weight between monozygotic twins to different adult health outcomes [Bo *et al*, 2000; Poulsen *et al*, 1997].

1.3.2 The concept of programming

Closely associated with the idea that the early life environment influences adult health, is the concept of 'programming'. Lucas [1991] is usually credited with coining the term, and he defined it as a "permanent or long-term change in the structure or function of the organism" resulting from an "early stimulus or insult, operating at a critical or sensitive period". He proposed that it could be induced either as part of normal development or in response to non-physiological events, and that it could be triggered by internal factors, such as the genetically-determined release of a hormone or growth factor, or by external stimuli. The term is often used with prefixes such as 'fetal', 'developmental' or 'prenatal' to describe programming at different stages of development, while the phrase 'nutritional programming' deals with the effects of periconceptional, prenatal, lactational and infant nutrition.

1.3.3 Evolutionary explanations

James Neel [1962] made the first attempt to explain programming or, more generally, the proposed link between the early environment and adult health, in evolutionary terms. His 'thrifty genotype' hypothesis suggested that so-called 'thrifty' genotypes that processed food more efficiently were selected for in times of limited nutrient availability, but that

this adaptation was disadvantageous in periods of adequate or excessive nutrition, and led to insulin resistance and T2DM. The incidence of metabolic disease was therefore proposed to be a combination of heritability and adult environment.

In contrast, Hales and Barker [1992] developed their now classic 'thrifty phenotype' hypothesis, which suggested that poor fetal or infant nutrition caused permanent changes to certain tissues and organs leading to functional and metabolic disorders in adulthood. Their idea was that this was an adaptive response by which the growth of certain tissues and organs was optimised at the expense of others in order to ensure the short-term survival of the organism, though possibly at the risk of longer-term health outcomes. They later refined their ideas to suggest that the adaptive response was not only to ensure immediate survival, but also to maximise the chances of survival in a 'predicted' postnatal environment. Thus, adaptations would only become detrimental if there was a 'mismatch' between the actual and predicted postnatal environment, such as when the offspring of a poorly-nourished mother were raised in conditions of abundant nutrition [Hales & Barker, 2001].

The latest iteration of DOHaD theory seeks to put the environmental influence on adult health and disease in the context of 'developmental plasticity', which has been defined as "the phenomenon by which one genotype can give rise to a range of different physiological or morphological states in response to different environmental conditions during development" [Barker, 2004]. Organisms respond to cues such as nutrition or hormones, and adapt their phenotype to their environment [Gluckman et al, 2010a]. These adaptations may either be homeostatic adaptive responses that provide an immediate survival benefit, non-adaptive responses that disrupt development (with possible adverse consequences in later life), or 'predictive adaptive responses' which are induced in expectation of adaptive advantages in a predicted future environment [Gluckman & Hanson, 2004]. In the last case, as proposed by Hales and Barker [2001], disease occurs if there is a mismatch between the predicted and realised environment [Gluckman et al, 2010a].

1.3.4 Biological effects and mechanisms

The biological consequences of programming include permanent changes to tissue and organ structure, mitochondrial functionality, and gene expression. Structural changes to a tissue or organ can reduce its serviceability in later life, mitochondrial dysfunction can lead to a build-up of reactive oxygen species and cumulative oxidative damage to proteins and DNA, and changes in gene expression can alter metabolic processes [Warner & Ozanne, 2010]. The exact mechanisms underlying such changes are not well understood [McMullen & Mostyn, 2009], although some changes occur simply because a lack of nutrients causes a

shortage of the raw materials required to build high-quality tissues and organs [Fall, 2011]. One potential mechanism is changes in the level of glucocorticoids, which stimulate tissue maturation by altering the balance between tissue growth and differentiation [Fowden *et al*, 1998], as there is now convincing evidence that suboptimal maternal nutrition increases fetal exposure to glucocorticoids [McMullen & Mostyn, 2009]. Another likely mechanism is the epigenetic regulation of gene expression. The term 'epigenetics' was coined in 1942 by Waddington [reprinted in Waddington, 2012] to describe the mechanisms by which genes bring about phenotypic effects. Although there is no generally accepted definition of the term *per se*, a meeting to address the issue in 2008 came up with a consensus definition for 'epigenetic trait', specifically "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [Berger *et al*, 2009]. The three main epigenetic mechanisms for regulating gene expression are DNA methylation (at the 5' position of cytosine in a cytosine-guanine dinucleotide) [Bird, 2002], histone modification (methylation, acetylation or phosphorylation of N-terminal histone tails) [Bannister & Kouzarides, 2011], and expression of non-coding RNAs (including microRNAs) [Mattick & Makunin, 2006]. Patterns of DNA methylation are probably established mainly in prenatal and early postnatal life [Burdge *et al*, 2007], and are potentially sensitive to early nutrition [Lillycrop *et al*, 2005; Waterland & Jirtle, 2003]. Furthermore, epigenetic modifications are heritable, and there is evidence from both human and animal studies that programming effects are not limited to a single generation [Drake & Walker, 2004; Painter *et al*, 2008].

1.4 Nutritional programming of obesity

1.4.1 Introduction

While the DOHaD hypothesis covers all environmental influences on early development, nutrition is clearly one of the most important. Indeed, it is now well established that early life nutrition is a factor in determining the risk of a range of adult diseases, including CVD, T2DM, asthma, lung disease, osteoporosis and some forms of cancer [BMA, 2009]. There is also now strong evidence from human and animal studies that suboptimal early nutrition can programme for adult obesity [Fall, 2011]. Furthermore, this outcome can be achieved by either a deficit or an excess of nutrients in the fetal period, though the precise timing of any nutritional challenge is a critical factor, and the effects can be sex-specific. An excess of nutrients and rapid growth in the early postnatal period can also have an effect on adult obesity, either *per se*, or in combination with prenatal over- and undernutrition.

1.4.2 Fetal undernutrition

Fetal undernutrition occurs when the fetal demand for nutrients, which largely depends on its rate of growth, exceeds its supply. It can arise as a result of inadequate maternal nutrition (in terms of total energy or specific nutrients), inability of the mother to mobilise and transport sufficient nutrients, or impairment of the fetal supply line [Fall, 2011].

1.4.2.1 Human studies

Human studies of fetal undernutrition are almost invariably retrospective cohort studies, either of populations from known famines, where maternal undernutrition can be reliably assumed, or of other populations, where low birth weight is used as a proxy for it. Some of the most convincing evidence that fetal undernutrition programmes for adult obesity was obtained from studies of the 'Dutch Hunger Winter' in the last months of World War II. A strict food embargo imposed by the German occupying forces in the western Netherlands during that period caused a severe short-term famine in the region. In an historical cohort study, Ravelli *et al* [1976] researched the records of around 308 000 19 year-old men who had been medically examined on call-up for national service between 1964 and 1967. It was found that rates of obesity were dependent on time of exposure to the famine: exposure in the first two trimesters of pregnancy produced significantly higher obesity rates in famine victims than in non-exposed controls, whereas exposure in the last trimester and first few months of postnatal life produced significantly lower rates. Another study of 741 subjects looked at the effect of prenatal exposure to the Dutch famine on obesity in 50 year-old men and women [Ravelli *et al*, 1999]. In this case, it was found that exposure to the famine in early gestation was associated with higher body weight, BMI and waist circumference in women, but not in men. Interestingly, given that birth weight is often used as a proxy for intrauterine nutritional conditions, the birth weight of subjects exposed to the famine in early gestation was not affected, whereas the birth weight of subjects exposed in mid- to late gestation was lower than that of non-exposed controls.

Further evidence is provided by the Great Chinese Famine, which occurred from the late 1950s to early 1960s. Various epidemiological studies have examined the relationship between famine exposure in early life and the risk of obesity and metabolic diseases in adulthood among different Chinese populations [reviewed in Li *et al*, 2013]. These showed consistent associations between fetal famine exposure and T2DM, hypertension and the metabolic syndrome in adulthood. Interestingly, and as observed in the study on the Dutch famine by Ravelli *et al* [1999], an association between fetal famine exposure and the risk of adult obesity was only consistently reported in women.

The results obtained from famine studies have not always been so consistent. A study of the severe famine during the siege of Leningrad between 1941 and 1944 found that there was no difference between exposed and non-exposed subjects in the level or centrality of adult obesity [Stanner et al, 1997]. One possible reason for the discrepancy between these results and those of the Dutch study is a difference in the postnatal nutritional conditions. The Dutch famine was much shorter and, unlike the Leningrad famine, adequate nutrition was restored almost immediately after it had ended. According to the DOHaD hypothesis, the nutritional mismatch during early childhood following the Dutch famine would have increased the risk of adverse health and obesity in adulthood [Stanner & Yudkin, 2001].

Finally, a number of other cohort studies have shown an association between low birth weight and abdominal fat deposition. For example, in a study of 845 men born in Hertfordshire from 1920 to 1930 (mean age 64) and 239 men born in Preston from 1935 to 1943 (mean age 51), Law et al [1992] found that central fat, as measured by WHR, was inversely correlated to birthweight after correcting for adult BMI. However, as was shown in the Dutch study [Ravelli et al, 1999], low birth weight is not necessarily a good indicator of fetal undernutrition, which limits the usefulness of human epidemiological studies.

1.4.2.2 Animal studies

A number of animal models have been used to test the programming effects of fetal undernutrition on subsequent obesity, including rodents, pigs and sheep. Methods of achieving fetal undernutrition include maternal diet manipulation, glucocorticoid infusion, uterine ligation, placental reduction, and utilisation of natural birth weight variation in litters [Fall, 2011; Mostyn & Symonds, 2009]. Manipulation of the maternal diet usually involves restricting either the total energy or protein content.

Numerous studies in rats and mice have found that protein [Bol et al, 2009; Ozanne et al, 2004; Zambrano et al, 2006] and calorie [Anguita et al, 1993; Jones et al, 1984; Vickers et al, 2000] restriction during gestation programmes for adult obesity, often in combination with a postnatal hypernutritional challenge, though there are inconsistencies between studies, some results are sex-specific [Jones et al, 1984; Zambrano et al, 2006], and calorie restriction seems to be more potent than protein restriction [Bieswal et al, 2006]. Indeed, one study found no clear evidence of obesity in rats at 18 months of age after protein restriction at different stages of, or throughout, pregnancy, although in this case there was no postnatal challenge [Bellinger et al, 2006]. The duration of nutrient restriction was usually the whole of gestation, though in some studies it was the first two weeks only (term = 21 days). In most cases, offspring of protein- and calorie-restricted dams had lower birth weights than controls.

Studies in pigs have mainly looked at low birth weight animals which occur naturally as a result of asymmetric intrauterine growth restriction (IUGR) in large litters. A number of such studies have shown that low birth weight piglets, or 'runts', grow more slowly and become fatter as adults than medium or high birth weight animals [Poore & Fowden, 2004; Powell & Aberle, 1980; Rehfeldt *et al*, 2008]. However, one study that specifically looked at the effect of maternal undernutrition (60 % of requirements) from mating to mid-gestation (term = 114 days) found no difference between the offspring of underfed and control sows in birth weight or, following a standard postnatal diet, relative adipose tissue at slaughter (105 kg) [Bee, 2004]. A more recent study looked at the effect of maternal undernutrition (50 % of requirements) throughout gestation or from day 36 of gestation, though it is not clear whether the restriction was continued into the lactation period. Offspring of sows undernourished in the last two trimesters (late underfed) had lower birth weight than offspring of controls or sows underfed throughout gestation. After weaning, piglets were fed a standard diet until 120 days of age followed by an obesogenic diet up to 240 days of age. From 90 days of age onwards (ie before exposure to the obesogenic diet), offspring of sows underfed throughout gestation had higher body weight, BMI and subcutaneous back fat than offspring of controls or late underfed sows, with the effects amplified in females. Furthermore, the female offspring of late underfed sows were also particularly prone to obesity, especially when exposed to the obesogenic diet [Barbero *et al*, 2013]. Finally, a study that looked at the effect of maternal protein restriction throughout gestation found that the offspring of mothers who were exposed to the low protein diet were fatter than controls by 188 days of age [Rehfeldt *et al*, 2012].

Numerous sheep studies, many of them recently reviewed and summarised by Kenyon and Blair [2014], have looked at the effect of maternal undernutrition at various stages of gestation on fetal growth, birth weight and weaning weight, though relatively few have studied long-term body weight and adiposity. The period of placental growth in sheep is from early to mid-gestation (30 to 80 days), while the period of maximum fetal growth is late gestation (90 days to term at 147 days) [Symonds *et al*, 2007]. In general, although there were exceptions, maternal undernutrition in early to mid-gestation had no effect on birth weight, while fetal undernutrition in late gestation reduced it [Kenyon & Blair, 2014]. This is consistent with the results from the Dutch famine (Section 1.4.2.1, p 39). The picture with respect to adiposity is more confused. Some studies of undernutrition in early to mid-gestation showed increased adiposity in postnatal life [Bispham *et al*, 2003; Ford *et al*, 2007; Zhu *et al*, 2006], while others found no effect [Gardner *et al*, 2005; Gnanalingham *et al*, 2005; Gopalakrishnan *et al*, 2004]. There are fewer studies of undernutrition in late gestation, but these have found reduced adiposity [Budge *et al*, 2004], no difference [Gnanalingham *et al*,

2005], or increased adiposity [Gardner *et al,* 2005]. One study showed either no difference or reduced adiposity, depending on the duration of the nutritional restriction [Oliver *et al,* 2001]. It is also worth noting, however, that studies of IUGR resulting in low birth weight, a condition often associated with undernutrition in late gestation, have consistently showed increased postnatal adiposity. In these studies, low birth weight was achieved naturally [Greenwood *et al,* 1998], by twinning and placental embolisation [Louey *et al,* 2005], or by placental restriction [De Blasio *et al,* 2007]. Although a review by Greenwood *et al* [2010], cited by Kenyon and Blair [2014], proposed that either prolonged severe growth restriction resulting in low birth weight, or prolonged severe undernutrition in late gestation, would predispose sheep to fatness in later life, it is difficult to draw such definitive conclusions from the disparate results set out above. While the precise timing of nutritional restriction is clearly a factor, likely confounding factors are the severity of restriction, fetal number, sex, the level and content of postnatal nutrition, particularly during lactation [Greenwood *et al,* 2010], and possibly also the breed.

1.4.3 Fetal overnutrition

1.4.3.1 Introduction

Fetal overnutrition is the oversupply of nutrients to the fetus. It is a function of maternal obesity, maternal overnutrition, often manifested as excess weight gain during pregnancy, or maternal diabetes. It is of particular significance because the recent global rise in overweight and obesity (Section 1.1.3, p 19) is mirrored in an increasing prevalence of maternal obesity and diabetes. A retrospective analysis of 287 213 pregnancies in an area of London in 1989–1997 found that 27.5 % of mothers were overweight and 10.9 % were obese [Sebire et al, 2001], and a more recent UK study found that 5 % of women who gave birth in or after the 24th week of gestation had a BMI of 35 or higher [CMACE 2010]. Similarly, a study in nine states of the USA found that pre-pregnancy obesity increased 69 % in 10 years, from 13 % in 1993–94 to 22 % in 2002–03 [Kim et al, 2007]. Maternal diabetes can be either pre-existing diabetes, present at the onset of pregnancy, or gestational diabetes (GDM). The latter usually develops in the third trimester of pregnancy and disappears soon after parturition [NHS, 2016]. It arises in some 8.8 % of pregnancies in the western world, and the risk of developing it is directly proportional to maternal BMI [Armitage et al, 2008]. Diabetic mothers are not only hyperglycaemic, but also have high levels of circulating amino acids and lipids [Fall, 2011].

1.4.3.2 Human studies

Obesity in pregnancy is associated with large for gestational age (LGA) babies [Surkan *et al*, 2004; Yu *et al*, 2013], and babies of obese mothers have a higher percentage of body fat than those of average or lean mothers [Catalano et al, 2009; Sewell et al, 2006]. A number of epidemiological studies have found that high birth weight [Curhan *et al*, 1996a; Curhan *et al*, 1996b; Parsons *et al*, 1999; Parsons *et al*, 2001] and maternal obesity [reviewed in Oken, 2009] are linked to childhood and adult BMI. Several studies have also found that maternal weight gain during pregnancy is associated with LGA babies and childhood [reviewed in Oken, 2009] or adult [Lawlor *et al*, 2011a] obesity, independently of BMI. Many studies have also shown that the offspring of diabetic mothers are LGA [Ehrenberg *et al*, 2004], and have a higher risk of obesity in later life [Dabelea *et al*, 2000; Gillman *et al*, 2003; Lawlor *et al*, 2011b; Pettitt *et al*, 1987]. This effect is also independent of BMI, though it can be difficult to differentiate between the effects of maternal obesity and diabetes, as they often occur together. In one study, the combination of maternal obesity and GDM was found to pose the greatest risk of offspring adiposity at 16 years of age, while the risk associated with GDM itself was small [Pirkola *et al*, 2010].

While there is clear evidence from human studies that maternal obesity, weight gain and diabetes are linked to childhood and adult obesity, an intrauterine programming effect cannot be assumed. It may be that the association arises from shared genes and/or familial environment. Indeed, it has been found that offspring obesity is also linked to paternal obesity, though the maternal effect is stronger than the paternal effect, supporting the case for an intrauterine influence on offspring adiposity [Whitaker *et al*, 2010]. It is also possible that some results are confounded by early postnatal nutrition. Breastfeeding is known to have a protective effect against obesity [Owen *et al*, 2005], and there is evidence that, in some populations, higher maternal BMI is associated with earlier termination of breastfeeding [Baker *et al*, 2007]. However, offspring of mothers who have gained weight between pregnancies have a greater risk of being born LGA than their siblings [Villamor & Cnattingius, 2006], and offspring of mothers who have had bariatric surgery to reduce their weight have a reduced risk of obesity compared with their siblings born before the surgery [Smith *et al*, 2009]. Furthermore, it has been found that the risk of obesity is higher in siblings born after a mother developed diabetes than in those who were born before the diagnosis, but that paternal diabetes has no effect on offspring obesity risk [Dabelea *et al*, 2000]. All these results support a nutritional programming effect.

1.4.3.3 Animal studies

Animal studies across several species have provided clear evidence linking maternal over-nutrition with an increased risk of offspring obesity. Most such studies have been carried out on rodents, for which either a purified high-fat diet (HFD) or a so-called 'cafeteria' diet is normally used [Li *et al*, 2011]. The composition of a cafeteria or 'junk food' diet varies, but usually includes a selection of readily-available junk foods that are typically energy-dense, nutrient-poor, and relatively high in fat, sugar and salt [Muhlhausler & Gugusheff, 2016]. A particular problem with most rodent studies, however, is that maternal overnutrition is continued into the lactation period, so it is difficult to isolate the fetal effect. A recent systematic review of rat models of a maternal HFD found it was a "real risk" for obesity and T2DM in the offspring [Ainge *et al*, 2011]. However, of the 15 datasets (from 11 different studies), 10 continued the HFD into the lactation period. One consistent feature, though, was that in 10 of the 11 studies there was no difference in birth weight between the HFD-fed and control groups, which contrasts with the findings from human studies (Section 1.4.3.2, p 43). Similarly, a maternal junk food diet during pregnancy and lactation in both rats [Nivoit *et al*, 2009] and mice [Samuelsson *et al*, 2008] has been found to increase obesity in adult offspring weaned onto a control diet. Another study in rats showed a similar effect when offspring were weaned onto a junk food diet, but in this case the increased obesity was more pronounced in females [Bayol *et al*, 2008].

A few studies have used cross-fostering during lactation to separate the programming effects of the fetal and lactational periods. One such study in rats using an HFD found that, when weaned onto a control diet, male offspring subjected to fetal overnutrition had more abdominal adiposity than controls at 180 days of age, whereas in females the effect was only observed if the overnutrition was continued into lactation [Khan *et al*, 2005]. Another study that, unusually, used a maternal high-protein diet found that male offspring exposed to excess protein prenatally had increased absolute and relative fat mass at 9 weeks of age (female offspring were not tested) [Daenzer *et al*, 2002]. More recent studies in mice using HFDs have shown contrasting results. One study found that both male and female offspring exposed to the HFD prenatally were heavier and had more fat than controls by around 12 weeks of age [Masuyama & Hiramatsu, 2014]. Another study found that male offspring of HFD-fed dams had higher body weight and body fat than controls from around 10 and 6 weeks of age respectively. In female offspring, however, there was no difference in body weight between the two groups but, very surprisingly, female offspring of HFD-fed dams had less body fat than controls from 16 weeks of age [Dahlhoff *et al*, 2014]. Finally, other studies in rodents have found that maternal obesity alone, without overnutrition during

gestation, also programmes for offspring obesity [Shankar *et al*, 2008] and, conversely, that preconceptional obesity is not a requirement for the programming of offspring obesity if the mother is exposed to an HFD during pregnancy and lactation [Howie *et al*, 2009].

There have been very few studies of fetal overnutrition in pigs. One study that looked at the effect of maternal overnutrition (140 % of requirements) from mating to mid-gestation found that there was no difference between groups in birth and weaning weights, but that the offspring of overnourished sows had more relative adipose tissue at slaughter (105 kg) than the offspring of controls [Bee, 2004]. Another study examined maternal overnutrition (160 % of requirements) for the whole of gestation (and possibly lactation). From 90 days of age, the offspring of overfed mothers had higher body weight, BMI and subcutaneous back fat than the offspring of controls, with more effect in females [Barbero *et al*, 2013]. Finally, a study that looked at the effect of excess maternal protein (250 % of requirements) throughout gestation found that the offspring of sows fed the high-protein diet had lower birth weight than the offspring of controls, but that at 188 days, following a standard postnatal diet, there was no difference between the groups in body weight or absolute or relative adipose tissue [Rehfeldt *et al*, 2011; Rehfeldt *et al*, 2012].

While there have been a number of studies of maternal overnutrition in sheep, most have looked at its effect on metabolism and physiological systems in the offspring rather than obesity *per se*. A typical study uses control and experimental groups fed to 100 % and 150–160 % of metabolisable energy requirements respectively, although the duration and timing of overnutrition vary. In most studies, in contrast to humans (Section 1.4.3.2. p 43), but similarly to rodents, maternal overnutrition does not have an effect on offspring birth weight or near-term fetal weight, irrespective of whether the overnutrition is applied before conception only [Rattanatray *et al*, 2010], from pre-conception to late gestation or term [Ford *et al*, 2009; Long *et al*, 2010; Yan *et al*, 2010; Zhang *et al*, 2011; Zhu *et al*, 2009], or in late gestation only [Muhlhausler *et al*, 2006]. However, overnutrition from mid-gestation to term or near-term has been reported to increase offspring birth or fetal weight [Bielli *et al*, 2001; Budge *et al*, 2000].

A few sheep studies have reported on adiposity, but only one has looked at the effect of maternal overnutrition on adult offspring. In this study, ewes in the experimental group were overfed from 60 days before conception to parturition. All offspring were fed on a standard diet until 19–20 months of age, at which point they were subjected to a 12-week *ad libitum* feeding challenge. There was no difference in body weight or fat mass between groups at the start of the challenge. However, by the end of the challenge, the offspring of overfed mothers had more body fat and less lean mass than the offspring of controls, with a "tendency" for increased body weight gain [Long *et al*, 2010]. Other studies have found

that offspring of overfed mothers had reduced total and relative perirenal adipose tissue at near-term (overnutrition from mid- to late gestation) [Budge *et al,* 2000], a "markedly" higher percentage body fat at birth (overnutrition from pre-conception to term) [Ford *et al,* 2009], increased relative subcutaneous, but not perirenal, adipose tissue at 30 days of age (overnutrition in late gestation) [Muhlhausler *et al,* 2006], and a higher total, but not relative, body fat mass in females, but not males, with the greatest impact on the visceral fat depots, at 4 months of age (overnutrition before conception) [Rattanatray *et al,* 2010]. Finally, one study has found that the female offspring of ewes provided with a high level of a micronutrient, selenium, throughout gestation were heavier at birth, and had more peri-renal adipose tissue at 180 days of age, than the offspring of ewes provided with only an adequate level (male offspring were not tested) [Vonnahme *et al,* 2010].

1.4.4 Early postnatal nutrition

Early postnatal nutrition, particularly during the suckling period, can also programme for adult obesity, often in combination with prenatal nutrition. One of the highest risks of adult obesity is fetal undernutrition followed by a nutritional mismatch in the lactation period, such as restoration of normal nutrition. Where fetally undernourished offspring are born small for gestational age (SGA), access to an adequate or excessive diet during the lactation period induces a phenomenon termed 'catch-up growth', which is an accelerated phase of growth that occurs after a transient period of nutrient restriction at any stage of development [Boersma & Wit, 1997]. The programming effects of this interaction between fetal undernutrition and a standard diet during lactation have been observed in rodents, both for low-energy [Desai *et al,* 2005; Vickers *et al,* 2000] and low-protein [Bol *et al,* 2009; Zambrano *et al,* 2006] diets, pigs [Rehfeldt *et al,* 2012] and sheep [Ford *et al,* 2007; Gardner *et al,* 2005]. Some studies have required an obesogenic diet after weaning to detect increased adiposity [Bol *et al,* 2009], while others have found that a standard diet is sufficient, but that the effect is amplified by an obesogenic diet [Bieswal *et al,* 2006]. Studies in rodents have also shown that if the nutritional restriction is maintained throughout gestation and lactation, the risk of adult obesity is reduced, and catch-up growth is attenuated or delayed [Bieswal *et al,* 2006; Desai *et al,* 2005; Jimenez-Chillaron *et al,* 2006]. Furthermore, it seems that, in rodents at least, undernutrition during the lactation period alone has no effect on adult obesity [Desai *et al,* 2005; Jimenez-Chillaron *et al,* 2006; Ozanne *et al,* 2004]. Finally, it has been shown in rodents that the programming effects of fetal undernutrition on adult obesity can be reversed if undernourished offspring are treated with the hormone leptin (Section 1.8.3.5, p 58) in the early postnatal period [Vickers *et al,* 2005], reinforcing the idea that the window for developmental plasticity continues into the early postnatal period.

In contrast to undernutrition, overnutrition during the suckling period alone does seem to programme for later obesity. In a human study, a protein-enriched formula milk fed to infants for the first year of life resulted in a higher weight gain by 2 years of age than breast milk or a control formula milk [Koletzko *et al*, 2009]. Furthermore, breastfeeding has been found to be protective against childhood and adult obesity [Arenz *et al*, 2004; Owen *et al*, 2005], an effect that may be attributable to a higher energy density or an excess of specific nutrients in formula milk. Studies in rodents using various techniques, including cross-fostering to dams on an HFD [Khan *et al*, 2005; Masuyama & Hiramatsu, 2014], litter size reduction [Plagemann *et al*, 1999; Plagemann *et al*, 1992], or artificial rearing on a high-carbohydrate formula milk [Srinivasan *et al*, 2008],[9] have also found that overnutrition in the lactation period, followed by weaning onto a standard diet, can programme for adult obesity. Furthermore, the combination of fetal and lactational overnutrition may have an additive effect [Khan *et al*, 2005; Masuyama & Hiramatsu, 2014]. Finally, the window for the programming of adult obesity by postnatal overnutrition is not confined to the suckling period. Numerous studies have found that rapid postnatal growth during early childhood (up to 2 years of age) is associated with adult obesity [reviewed in Ong & Loos, 2006]. This is not limited to catch-up growth following fetal undernutrition, as the effect is observed in both SGA and average birth weight offspring.

1.4.5 Mechanisms

The precise mechanisms for the nutritional programming of adult obesity are unclear, but are likely to involve permanent changes to pathways, organs or tissues that act to increase energy intake, reduce energy expenditure, or alter the balance and distribution of fat and lean mass. These effects could be mediated by changes in appetite, food preferences and satiety, by a reduction in physical activity (via muscle impairment or altered behaviour), by changes in energy metabolism, such as a reduction in BMR or enhanced thermogenesis, or by changes in adipose tissue development, composition, distribution and functionality. All of these proposed mechanisms would require changes in gene expression [Fall, 2011; Taylor & Poston, 2007]. There is evidence from animal models that at least some of these effects can be programmed by suboptimal early nutrition. In rodents, for example, both fetal/lactational overnutrition [Nivoit *et al*, 2009; Samuelsson *et al*, 2008] and fetal under-nutrition [Desai *et al*, 2005; Vickers *et al*, 2000] have been found to increase food intake (hyperphagia), and fetal overnutrition in sheep has been found to alter development of the appetite-regulating network of the brain [Muhlhausler *et al*, 2006]. Maternal overnutrition

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[9] Artificial rearing on a high-fat formula milk did not induce adult obesity [Srinivasan *et al*, 2008].

in rats and mice [Khan *et al*, 2003; Samuelsson *et al*, 2008], and undernutrition in rats and sheep [Donovan *et al*, 2013; Vickers *et al*, 2003], have also been found to reduce locomotor activity in the offspring.

1.4.6 Programming of adipose tissue

In humans, adipogenesis takes place during late fetal and (relatively) early postnatal life [Muhlhausler & Smith, 2009], and adipocyte number, but not size, is set in childhood and adolescence [Spalding et al, 2008]. Adipose tissue continues to grow for the first year of life through an increase in adipocyte size (hypertrophy), then declines for a year or so before stabilising. At around 6 years of age, it rapidly starts growing again through a combination of increased cell number (hyperplasia) and hypertrophy, the so-called 'adiposity rebound'. An early adiposity rebound (< 5.5 years) is associated with adult obesity, irrespective of the level of adiposity at 1 year of age [Rolland-Cachera *et al*, 1984].

As posited in Section 1.4.5, p 47, nutritional programming of obesity could be mediated by changes to the development (timing of initial growth, adiposity rebound), composition (cell number and size, WAT versus BAT), distribution (visceral versus subcutaneous depots) and functionality (thermogenic capacity, ability to expand and contract) of adipose tissue. There is evidence from animal models for some of these proposed mechanisms. In sheep, for example, which have a similar fat development profile to humans, nutrient restriction in early to mid-gestation [Bispham *et al*, 2003], and overnutrition from pre-conception to term [Ford *et al*, 2009], have been found to increase deposition of adipose tissue at term, while nutrient restriction in late gestation [Symonds *et al*, 1998], and overnutrition in mid-to late gestation, have been found to reduce it [Budge *et al*, 2000]. A cafeteria diet during gestation and lactation in rats has been found to increase adipocyte hypertrophy, but not hyperplasia, at weaning [Bayol *et al*, 2005], while moderate calorie restriction from early to mid-gestation in rats has been found to increase adipocyte hypertrophy in the inguinal depot and hyperplasia in the retroperitoneal depot of 6 month-old male offspring [Garcia *et al*, 2011]. There is also evidence from sheep and rats respectively that fetal overnutrition [Budge *et al*, 2000] and undernutrition [Palou *et al*, 2015] can alter thermogenic activity or capacity, at least at term or in early postnatal life.

All these potential mechanisms are likely to be induced by changes in the expression of adipogenic, lipogenic, thermogenic, structural and other genes in adipocytes. For example, a series of studies in rats has found that both fetal over- and undernutrition increase the expression of the principal regulator of adipogenesis, PPARγ (Section 1.8.2.3, p 54), albeit by different mechanisms [Desai & Ross, 2011]. Similarly, late gestational undernutrition has been found to increase the expression of PPARγ in the fetal adipose tissue of near-term

sheep [Muhlhausler *et al,* 2007]. In contrast, undernutrition in early to mid-gestation had no effect on PPARγ expression in fetal adipose tissue at the same time point [Bispham *et al,* 2005], while a study in rats found that a maternal low-protein diet throughout gestation reduced PPARγ expression in the offspring at 34 days of age [Burdge *et al,* 2004]. In sheep, fetal overnutrition from mid- to late gestation has been found to increase expression of the key thermogenic gene, UCP1 (Section 1.8.4.6, p 63), in fetal adipose tissue at near-term [Budge *et al,* 2000]. Maternal undernutrition from pre-conception to term also increased the expression of UCP1 mRNA (but not protein) in the fetal adipose tissue of sheep at near-term [Budge *et al,* 2004], while fetal undernutrition in late gestation reduced it [Budge *et al,* 2004], and fetal undernutrition from early to mid-gestation had no effect on its expression [Bispham *et al,* 2005]. It seems, therefore, that the timing and type of nutritional challenge is as much a factor in the modulation of early gene expression in adipose tissue as it is in the development of adult obesity itself.

1.4.7 Summary

Overall, evidence from animal studies suggests that fetal and early postnatal overnutrition, and fetal undernutrition, programme for adult obesity. Results of human epidemiological studies present a similar picture, albeit largely based on anthropometric data. Curhan *et al* [1996a] first proposed that the relationship between birth weight and adult BMI was U-shaped, while a later review of over 400 papers concluded that there is good evidence of an association between birth weight and subsequent BMI in children and young adults, which is linear and positive in some studies, and J- or U-shaped in others (although the evidence was less convincing for middle-aged subjects) [Rogers & the Euro-BLCS Study Group, 2003]. In both human and animal studies, the timing of the nutritional challenge is an important factor, probably reflecting the different stages of development: embryonic, placental and fetal. It is also apparent that nutritional programming can be sex-specific. However, results are often inconsistent, probably as a result of differences in study design and/or confounding variables. There are various potential mechanisms for the nutritional programming of obesity, some of which involve adipose tissue biology. These are probably mediated by changes in gene expression, and indeed there is evidence from animal studies that maternal malnutrition can alter the expression of adipogenic and thermogenic genes in fetal adipose tissue.

1.5 Sheep as a model for human obesity

For ethical reasons, it is not possible to use humans in invasive experiments, and their role as a subject of obesity research is largely limited to epidemiological studies. Animal models are therefore used to investigate the physiological mechanisms underlying human obesity. Rats and mice have been widely used for studies of obesity, adipose tissue and metabolism, as they are cheap to house and feed, they have relatively short gestation periods (~21 days) and lifespans, transgenic strains are readily available, and it is easy to manipulate their diet and environment. However, their short gestation periods and large litters do not make them particularly good models for human development. Furthermore, rodents are altricial, and their central nervous systems and hypothalamic-pituitary-adrenal (HPA) axes are not as mature at birth as those of precocial species or humans [McMullen & Mostyn, 2009; Romijn *et al*, 1991]. Additionally, deposition of BAT primarily takes place postnatally in rodents, with its main depot being the interscapular, whereas in humans and other large mammals it occurs *in utero*, with its main depot being the perirenal [Cannon & Nedergaard, 2004; Lean, 1989]. Indeed, in contrast to large mammals, rodents have very little fat of any sort at birth [Symonds *et al*, 2015]. Finally, BAT makes up as much as 5–10 % of body weight in mice, being two orders of magnitude more than that of humans (0.05–0.5 %) [Celi *et al*, 2015].

There is no perfect animal model for human physiology, but it was felt that similarity to humans in terms of adipose tissue development, HPA maturity, gestation period, litter size and birth weight were the most important factors for this study, all of which point towards a large mammal model. The 'gold standard' for such a model is the non-human primate, because of its evolutionary similarity to humans. Indeed, non-human primates are the only group which have an equivalent of the distinctive and highly-selective human abdominal 'paunch' adipose tissue depot [Pond, 1998]. However, cost and ethical considerations rule out such a model, at least in the UK. Next in line is the pig, a monogastric with a relatively long gestation period of around 114 days, that has recently become a popular model for nutritional studies because of its physiological and genetic similarity to humans [Baker, 2008; Mitchell, 2007; Puiman & Stoll, 2008]. Its main disadvantages are that it has a large litter of up to 20 piglets of highly variable birth weight (0.6–3 kg) [McMullen & Mostyn, 2009] and that, at least until recently, it was not considered to have any BAT because of a disrupted UCP1 gene [Berg *et al*, 2006; Trayhurn *et al*, 1989].[10] It was felt that for this study its disadvantages outweighed its advantages, and a sheep model was ultimately selected.

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[10] Surprisingly, a very recent study has found that UCP1 is present in the perirenal adipose tissue of newborn piglets, and that it is responsive to endocrine stimulation [Mostyn *et al*, 2014].

The sheep has been an established model in neonatology since the 1960s. Its benefits as a model for human development are that it is a large, precocial mammal with a relatively long gestation period (term = 147 days) that produces between one and three offspring of a similar birth weight (3–5 kg) to humans. Furthermore, its rapid growth of adipose tissue in late gestation, its pattern of development, distribution, activation and atrophy of BAT, and the maturity of its HPA axis at birth, are similar to those of humans [McMullen & Mostyn, 2009; Symonds *et al*, 2007]. Its disadvantages include time and cost, with the latter having an obvious impact on sample size, and therefore statistical power. However, its principal disadvantage in relation to nutritional studies is that it is a herbivore and a ruminant, so it does not metabolise energy in the same way as a monogastric species such as a human. In the first weeks after birth, this is less of an issue, as the rumen is not fully developed [Lane *et al*, 2000], and in any event, suckling elicits the so-called oesophageal groove reflex [Comline & Titchen, 1951; Ørskov & Benzie, 1969], which enables most of the milk to bypass the rumen and flow directly to the abomasum (the equivalent of a monogastric stomach). It is more of an issue with older animals, but the problem can be mitigated to some extent, and this is considered further in Sections 5.3.1.4, p 198, and 6.6.1, p 236.

1.6 Sternal adipose tissue

It was explained in Section 1.2.2.6, p 28, that the two main BAT depots in adult humans are the supraclavicular and the neck. In 2012, our research group identified an adipose tissue depot in sheep, where the sternal and clavicular areas meet the forelimb, that is analogous to the supraclavicular depot in humans, and which had a higher relative UCP1 expression in three month-old lambs than the other depots tested [Symonds *et al*, 2012b]. This depot was designated the 'sternal/clavicular' or, more simply, the 'sternal', the latter term being used throughout this thesis. It was selected to be the principal tissue of investigation, as its comparability to the human supraclavicular depot, and relatively high UCP1 expression, made it the most promising site for the potential nutritional programming of BAT. The anatomical location of sternal adipose tissue, and some of the other adipose tissue depots in sheep, is shown in Figure 1.6, p 52.

1.7 Aims and hypotheses

The principal aim of this study was to investigate, using sheep as a model, the effect of early nutritional interventions on the growth of fat mass and the expression in (primarily sternal) adipose tissue of a selection of genes involved in adipogenesis, energy metabolism, thermogenesis and development, with a view to providing evidence to support the concept
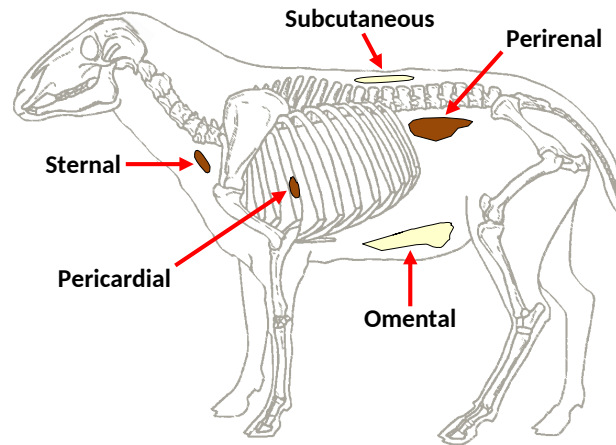
Figure 1.6 Anatomical location of some of the adipose tissue depots in sheep. The diagram is of a 3 month-old lamb, and is adapted from a figure in Symonds *et al* [2012b].

of the nutritional programming of obesity. A secondary aim was to study the ontogeny of fat mass and gene expression in sternal adipose tissue over the first month of postnatal life, which has not previously been characterised. A final aim, in the light of evidence that UCP1-expressing adipocytes in human supraclavicular fat are brite/beige [Wu *et al*, 2012], was to determine the nature of such cells in the sternal adipose tissue of sheep.

Specific hypotheses for different parts of the study are set out in Sections 3.2, p 131, 4.2, p 148, and 5.2, p 195. The overall, and more general, hypotheses were as follows:

- perinatal nutritional interventions would affect the adipose tissue mass of infant and adolescent lambs;

- perinatal nutritional interventions would change the expression of adipogenic, metabolic, thermogenic and developmental genes in the adipose tissue of infant and adolescent lambs;

- any increase in expression of adipogenic and metabolic genes would be partially compensated for by an increase in expression of thermogenic genes;

- the ontogeny of gene expression in the sternal adipose tissue of newborn lambs in the first month after birth would be similar to that of perirenal adipose tissue;

- UCP1-expressing adipocytes in the sternal adipose tissue depot of sheep would be brite/beige rather than brown.

1.8 Genes of interest

1.8.1 Introduction

The selection of the genes for testing was driven by a desire to examine a representative sample of the principal genes involved in adipogenesis, lipid and glucose metabolism, and thermogenesis, but was also a function of primer limitations. For example, it was intended to test the early adipogenic transcription factor C/EBPβ and the classical BAT marker ZIC1, but primers for these genes could not be found or designed that were effective in the tissues examined.[11] An attempt has been made to classify each of the genes selected into one of the three categories, mainly for presentation purposes, but this was somewhat arbitrary where they are multifunctional. Finally, certain developmental genes that have been described as markers of white, brite/beige or brown adipose tissue were also tested, and have been grouped in a fourth category, along with the BAT fate-determining gene, PRDM16.

Detailed descriptions by category of all the genes of interest are given in Section 1.8.2, p 53 (adipogenic genes), Section 1.8.3, p 56 (metabolic genes), Section 1.8.4, p 59 (thermogenic genes) and Section 1.8.5, p 63 (developmental genes). A summary description of all the genes in alphabetical order is shown in Table 1.2, p 66.

1.8.2 Adipogenic genes

1.8.2.1 CCAAT/enhancer-binding protein (C/EBP) α

The transcription factor C/EBPα (Figure 1.5, p 34) is one of the two main regulators (along with PPARγ; Section 1.8.2.3, p 54) of terminal adipocyte differentiation. It functions synergistically with PPARγ in that both factors promote each other's expression in a positive feedback loop, and together induce the expression of many genes associated with mature adipocytes. They participate in a single developmental pathway, with PPARγ as the main effector, and the principal function of C/EBPα in adipogenesis seems to be the induction and maintenance of PPARγ expression [Rosen *et al,* 2002]. Curiously, although it is essential for WAT development in the adult, it is not required for embryonic adipogenesis [Wang *et al,* 2015] or BAT development [Linhart *et al,* 2001]. In addition to stimulating the expression of PPARγ, C/EBPα is critical in adipogenesis for establishing insulin sensitivity, in part by the direct induction of insulin receptor (INSR; Section 1.8.3.4, p 57) and insulin receptor

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[11] C/EBPβ, like most members of the C/EBP family, is a single-exon gene, which is not well-suited to primer design (Section 2.4.10, p 81).

substrate (IRS) 1 [El-Jack *et al*, 1999; Wu *et al*, 1999]. It also plays a role in the regulation of energy metabolism: in mature adipocytes, it promotes expression of the adipokines adipsin and leptin (Section 1.8.3.5, p 58) [Chen *et al*, 2000], and in the liver, where it is very highly expressed, it regulates several genes involved in glycogen synthesis and gluconeogenesis [Ramji & Foka, 2002]. Finally, it also regulates its own expression [Christy *et al*, 1991].

1.8.2.2 Glucocorticoid receptor (NR3C1)

Glucocorticoid receptor (GR; Figure 1.5, p 34) is an intracellular receptor that is encoded by the nuclear receptor (NR) 3C1 gene. It is a ligand-activated transcription factor that acts either directly, by binding to glucocorticoid response elements (GREs) on the promoters of target genes, or indirectly, by binding to and regulating other transcription factors such as nuclear factor (NF) κB. It is expressed in most cells, and mediates numerous physiological functions, including glucose and lipid metabolism and the inflammatory response. Its presence is essential for adipogenesis, where it acts primarily by promoting the expression of KLF15, which in turn induces the expression of PPARγ (Section 1.8.2.3, p 54) [Asada *et al*, 2011]. It also inhibits the expression and function of UCP1 (Section 1.8.4.6, p 63), and thus thermogenesis, in brown adipocytes [Viengchareun *et al*, 2001]. Its cognate ligands are glucocorticoids (GCs), such as cortisol in humans and corticosterone in rodents, though it can also bind to mineralocorticoids, such as aldosterone. GCs are steroid hormones that are released from the adrenal cortex in response to stress. They are potent inducers of adipogenesis, and the synthetic GC dexamethasone, like insulin, is a component of the hormonal cocktail that induces adipocyte differentiation *in vitro*. Finally, prolonged exposure to GCs promotes adiposity, particular in the visceral depots [Campbell *et al*, 2011; Lee & Fried, 2014; Lu *et al*, 2006].

1.8.2.3 Peroxisome proliferator-activated receptor (PPAR) γ

PPARγ (Figure 1.5, p 34) is a ligand-activated nuclear receptor and transcription factor that binds to PPAR response elements (PPREs) in the promoter regions of target genes as a requisite heterodimer with retinoid X receptor (RXR). Its gene has three splice variants, giving rise to two protein isoforms: PPARγ1 and PPARγ2. Both are produced in adipose tissue, though the former is also found in other tissues. PPARγ is activated by a variety of natural and synthetic ligands, including fatty acids, eicosanoids and thiazolidinediones (TZDs).[12] It has often been described as the 'master regulator' of adipogenesis, and so far no other transcription factor has been found that can promote adipocyte differentiation in its

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[12] TZD drugs are used for the treatment of T2DM.

absence. In addition to its essential role in WAT and BAT adipogenesis, it promotes free fatty acid (FFA) uptake and triglyceride storage in WAT by upregulating genes involved in: (1) the release of FFAs from lipoproteins and their uptake, intracellular transport (eg FABP4; Section 1.8.3.2, p 56), activation and esterification; and (2) lipid droplet formation (eg CIDEA; Section 1.8.4.2, p 60). It also inhibits proinflammatory transcription factors, thereby reducing inflammation in adipose tissue, and activates expression of the adipokine adiponectin (Section 1.8.3.1, p 56), which promotes FFA oxidation and insulin sensitivity in liver and muscle. It also plays a further role in BAT development in that TZDs and its other agonists strongly stimulate expression of UCP1 (Section 1.8.4.6, p 63) in BAT and promote the 'browning' of WAT. Furthermore, in BAT it promotes mitochondrial biogenesis and thermogenesis in a non-ligand dependent manner on binding to its coactivator PGC1α (Section 1.8.4.4, p 61). Finally, treatment with its agonists can cause redistribution of WAT in humans, specifically preferential deposition of fat in subcutaneous rather than visceral depots. In summary, it is an important regulator of adipogenesis, whole body lipid and glucose metabolism, and thermogenesis, and is therefore a major target for the treatment of obesity and diabetes [Christodoulides & Vidal-Puig, 2010; Siersbæk *et al*, 2010; Tontonoz & Spiegelman, 2008].

1.8.2.4 Sterol regulatory element-binding factor (SREBF) 1

SREBF1 is one of two genes that encode a family of three sterol regulatory element binding proteins (SREBPs) that regulate genes involved in the synthesis of cholesterol, fatty acids, triglycerides and phospholipids. It encodes two such proteins, SREBP1a and SREBP1c, under the control of different promoters. SREBP1c (Figure 1.5, p 34), the principal isoform in adipose tissue and liver, regulates lipogenesis and is induced by insulin [Kim *et al*, 1998a]. It has long been linked with adipogenesis, as suggested by its original name, adipocyte determination and differentiation dependent factor 1 [Tontonoz *et al*, 1993]. In spite of this, its role in adipogenesis is somewhat unclear, and indeed may not be critical. It is induced later in the differentiation process than PPARγ (Section 1.8.2.3, p 54) and C/EBPα (Section 1.8.2.1, p 53), some 20 hours after initiation [Tang & Lane, 2012], and although *in vitro* studies suggest that it enhances the adipogenic effect of PPARγ [Kim & Spiegelman, 1996] and contributes to the production of endogenous PPARγ ligands [Kim *et al*, 1998b], various *in vivo* studies indicate that it is not necessary for the creation or expansion of adipose tissue [White & Stephens, 2010]. SREBP1a is primarily regulated by intracellular cholesterol levels and is a more powerful transcription factor than SREBP1c [Eberlé *et al*, 2004]. Although historically it has not been considered important for adipogenesis, more recent studies have linked it to preadipocyte proliferation and adipogenesis *in vitro*, where

it may act upstream of PPARγ, C/EBPα and SREBP1c [Alvarez *et al*, 2014; Ayala-Sumuano *et al*, 2011].

1.8.3 Metabolic genes

1.8.3.1 Adiponectin (ADIPOQ)

Adiponectin (transcribed from the ADIPOQ gene) is one of a number of cytokines that are secreted almost exclusively by adipose tissue, and are therefore termed 'adipokines'. It is the most abundant peptide secreted by adipocytes [Maia-Fernandes *et al*, 2008; Silva *et al*, 2014] and, in contrast to other adipokines, notably leptin (Section 1.8.3.5, p 58), it is negatively correlated with obesity in adults [Arita *et al*, 1999] and children [Stefan *et al*, 2002]. However, in the newborn period it is positively correlated with BMI [Kotani *et al*, 2004; Tsai *et al*, 2004], and its plasma concentration increases in the first month after birth [Inami *et al*, 2007]. It increases insulin sensitivity, and plays an important role in controlling energy homeostasis through regulation of glucose and lipid metabolism in peripheral tissues such as liver and muscle. It has antiatherogenic and antiinflammatory properties, and a recent study has found that it also inhibits expression of UCP1 (Section 1.8.4.6, p 63) in BAT [Qiao *et al*, 2014]. It is regulated by the adipogenic transcription factors C/EBPα (Section 1.8.2.1, p 53), PPARγ (Section 1.8.2.3, p 54) and SREBP1c (Section 1.8.2.4, p 55), and its transcription is stimulated by the PPARγ agonists TZDs [Galic *et al*, 2010; Liu & Liu, 2010; Rosen *et al*, 2009]. It is also regulated by calorie intake, but not by dietary fat [Qiao *et al*, 2011].

1.8.3.2 Fatty acid binding protein (FABP) 4

FABPs are a family of small, highly-expressed, cytoplasmic chaperones that reversibly bind hydrophobic ligands such as long-chain fatty acids and eicosanoids. Their biological role and mechanism of action are not well understood [Maeda *et al*, 2005], but they appear to be involved in intracellular lipid transport, distributing ligands for storage, activation of enzymes, or signalling. FABP4, also known as adipocyte-fatty acid binding protein, adipocyte lipid binding protein and adipocyte protein 2, is mainly expressed in adipose tissue and macrophages, and is induced by both C/EBPα (Section 1.8.2.1, p 53) and PPARγ (Section 1.8.2.3, p 54). It is expressed at extremely high levels in mature adipocytes, making up some 1–3 % of soluble cytosolic protein [Coe & Bernlohr, 1998], and has therefore been extensively used as a marker of adipocyte differentiation [Shan *et al*, 2013]. Recent studies have suggested that it is a circulating protein [Xu *et al*, 2006], and may therefore also act as an adipokine, and that it negatively regulates adipogenesis by triggering the proteasomal degradation of PPARγ [Garin-Shkolnik *et al*, 2014].

1.8.3.3 G protein-coupled receptor (GPR) 120

GPR120, also known as free fatty acid receptor 4 and omega-3 fatty acid receptor 1, is a cell membrane-bound receptor for long-chain saturated (C_{14} to C_{18}) and unsaturated (C_{16} to C_{22}) fatty acids [Hirasawa et al, 2005]. It is particularly selective for omega (ω)-3 fatty acids, and mediates their antiinflammatory and insulin-sensitising effects, particularly in macrophages [Oh et al, 2010]. It is highly expressed in the intestinal tract, macrophages, pituitary gland, spleen, lungs, taste buds and adipose tissue [Gotoh et al, 2007; Hirasawa et al, 2005; Matsumura et al, 2007; Miyauchi et al, 2009; Oh et al, 2010], and it seems to mediate the fatty acid regulation of energy homeostasis. Its activation induces secretion of the incretins glucagon-like peptide 1 and cholecystokinin in the intestine [Hirasawa et al, 2005; Tanaka et al, 2008], and increases glucose transport and translocation of GLUT4 to the cell membrane in adipocytes [Oh et al, 2010]. In taste buds, it plays a role in mediating taste preference for fatty acids, and its knock-down reduces preference for linoleic and oleic acid in mice [Cartoni et al, 2010]. It also appears to regulate adipogenesis, as it is upregulated during differentiation of both murine and human adipocytes, and differentiation is inhibited when it is knocked down [Gotoh et al, 2007]. In spite of reduced adipocyte differentiation, mice deficient in it are more obese on a high-fat diet than wild types (presumably through hypertrophy), and develop glucose intolerance [Ichimura et al, 2012]. In the adipose tissue of humans, it is more highly expressed in obese than in lean individuals, and a deleterious non-synonymous mutation in its human gene that inhibits its signalling activity increases the risk of obesity [Ichimura et al, 2012]. Finally, it is potentially of interest with regard to thermogenesis, because it is upregulated in the BAT and subcutaneous WAT of mice on cold exposure, and it may therefore be a factor in the 'browning' of WAT [Rosell et al, 2014].

1.8.3.4 Insulin receptor (INSR)

Insulin receptor (IR) is a tetramer that consists of two extracellular α subunits and two transmembrane β subunits. It is a member of a subfamily of receptor tyrosine kinases that includes the very similar insulin-like growth factor 1 receptor (IGF1R). Each pair of α and β subunits is post-translationally cleaved from a single precursor peptide that is encoded by the INSR (also known as the IR) gene. Two of the α–β dimers are then linked by disulfide bonds to form the tetramer. An IR α–β dimer can also bind to an IGF1R α–β dimer to form a hybrid tetramer. Its ligands, usually insulin or IGFs, bind to the receptor complex and trigger a conformational change that leads to the recruitment and phosphorylation of various adapter proteins, such as the IRSs. This in turn leads to the activation of two main signalling pathways that (1) modulate energy metabolism and (2) regulate gene expression

and control cell growth and differentiation. In its classical role, insulin, released from pancreatic β-cells, binds to IR and mediates the translocation of GLUT4 from the cytoplasm to the cell membrane, to facilitate glucose uptake. Dysfunction of IR, the only element of the pathway which is unique to insulin action, can lead to insulin resistance and T2DM. Insulin is also a powerful inducer of adipogenesis, and it (or IGF1) is a key component of the hormonal cocktail used to initiate adipocyte differentiation *in vitro*. Although IR is only expressed at low levels in preadipocytes, where insulin signalling is principally mediated through IGF1R, its expression increases markedly during differentiation such that it is the predominant receptor in mature adipocytes. Mechanistically, its substrates IRS1 and IRS2 play a key role in the upregulation of PPARγ (Section 1.8.2.3, p 54) and C/EBPα (Section 1.8.2.1, p 53) [Miki *et al*, 2001; Ramalingam *et al*, 2013; Taniguchi *et al*, 2006; Youngren, 2007].

1.8.3.5 Leptin (LEP)

Leptin, which is transcribed by the LEP or OB (obese) gene, is an adipokine that is mainly synthesised in, and secreted by, white adipocytes. It plays an important role in energy homeostasis, particularly control of adipose tissue mass, and its mRNA, protein and plasma levels correlate to WAT mass. It also plays a role in reproduction and development, and in the regulation of the immune, cardiovascular and other systems. It acts through neurons in the brainstem and hypothalamus, notably the arcuate nucleus, and signals the body's nutritional state to various physiological systems. When its levels are low, it mediates the primary adaptive response to starvation and fasting by stimulating appetite and reducing energy expenditure. Its deficiency causes severe obesity, and leads to irregularities in glucose and lipid metabolism and in most physiological systems. When its levels are high, it usually triggers a reduction in food intake and an increase in energy expenditure, leading to a loss of adipose tissue mass. However, although its levels are high in obese humans, the homeostatic restorative mechanism seems to break down and there is no subsequent loss of weight, indicating the development of leptin resistance. It is first expressed in the later stages of adipogenesis when lipids start to form, induced by PPARγ (Section 1.8.2.3, p 54), the C/EBP family and other factors. Its expression is increased by endotoxins and infection, and by glucocorticoids, insulin and proinflammatory cytokines, and is reduced by cold exposure, thyroid hormones and catecholamines [Ahima & Flier, 2000; Friedman & Halaas, 1998].

1.8.3.6 Receptor-interacting protein (RIP) 140

RIP140, also known as nuclear receptor-interacting protein 1, is widely expressed, controls a number of physiological processes, and has a role in the heart, macrophages, mammary

gland and the brain. It is highly expressed in metabolic tissues such as liver, muscle and adipose tissue, where its principal role is as a ligand-dependent corepressor of nuclear receptors (eg PPARs, thyroid hormone receptors and oestrogen-related receptors) that are key regulators of energy homeostasis. It also binds directly to, and suppresses the activity of, the coactivator PGC1α (Section 1.8.4.4, p 61), which targets the same receptors [Hallberg *et al*, 2008]. By such means, it suppresses catabolic pathways such as fatty acid oxidation and glycolysis. It is not involved in adipogenesis, but its expression is induced during the differentiation process. In WAT, it inhibits expression of UCP1 (Section 1.8.4.6, p 63), CIDEA (Section 1.8.4.2, p 60) and other genes involved in thermogenesis, thus blocking activation of brite/beige adipocytes and preventing 'browning' of the tissue. It also has a role in the cytoplasm, where it suppresses GLUT4-mediated glucose uptake and adiponectin (Section 1.8.3.1, p 56) secretion [Ho *et al*, 2009; Ho & Wei, 2012]. Finally, it acts as a transcriptional coactivator in the regulation of such diverse process as circadian rhythms, inflammation, mammary gland development, and ovulation. RIP140-deficient mice show a phenotype of female infertility and impaired energy homeostasis. They are very lean (70 % less total fat mass), obesity-resistant on a high-fat diet, more insulin sensitive and glucose tolerant, and have higher UCP1 expression compared to wild types. They also have much smaller white adipocytes, even though their brown adipocytes are histologically normal [Christian *et al*, 2006; Leonardsson *et al*, 2004; Nautiyal *et al*, 2013].

1.8.4 Thermogenic genes

1.8.4.1 Activating transcription factor (ATF) 2

ATF2 (Figure 1.3, p 26) has been termed a 'moonlighting' protein [NCBI, 2014a] because it has multiple functions, including histone acetylation [Bruhat *et al*, 2007] and DNA damage response [Bhoumik *et al*, 2005]. It is a member of the CREB family of factors, and plays a role in β_3-adrenergic activation of thermogenesis in BAT by promoting expression of the thermogenic genes UCP1 (Section 1.8.4.6, p 63) and PGC1α (Section 1.8.4.4, p 61) [Cao *et al*, 2004; Collins *et al*, 2010]. It is also expressed in WAT and liver, where it influences glucose and lipid metabolism by promoting expression of phosphoenolpyruvate carboxykinase, an important enzyme in gluconeogenesis and glyceroneogenesis [Cheong *et al*, 1998; Lee *et al*, 2002]. It also plays a role in the early stages of adipogenesis, by acting in coordination with C/EBPβ to regulate expression of PPARγ (Section 1.8.2.3, p 54) [Lee *et al*, 2001; Maekawa *et al*, 2010]. Finally, it promotes the expression of proinflammatory genes, including tumour necrosis factor (TNF) α, and is highly expressed in infiltrating macrophages [Brinkman *et al*, 1999; Yu *et al*, 2014].

1.8.4.2 Cell death-inducing DNA fragmentation factor α-like effector (CIDE) A

CIDEA is one of a family of proteins that were originally identified (and named) for their role in inducing cell death [Inohara *et al,* 1998]. In common with its closely-related family member CIDEC,[13] it is highly expressed in adipose tissue and plays an important role in energy metabolism. Its most clearly-defined role in adipose tissue is the enlargement of lipid droplets, in conjunction with CIDEC, by mediating the transfer of triglycerides from smaller to larger droplets [Gong *et al,* 2011; Puri *et al,* 2008; Wu *et al,* 2014]. However, its expression pattern varies between species. In mice, it is highly expressed in BAT,[14] but not expressed in WAT [Zhou *et al,* 2003].[15] In humans, on the other hand, it is highly expressed in WAT [Nordström *et al,* 2005]. Its expression in murine BAT is not influenced by obesity, while its expression is reduced in the WAT of obese humans and normalised after weight reduction [Nordström *et al,* 2005]. Furthermore, a single-nucleotide polymorphism in its human gene is a risk factor for obesity and the metabolic syndrome [Dahlman *et al,* 2005; Zhang *et al,* 2008], and its expression in human WAT is positively correlated with insulin sensitivity [Puri *et al,* 2008]. Its expression in murine BAT mirrors that of UCP1 (Section 1.8.4.6, p 63), but paradoxically it actually seems to inhibit thermogenesis by negatively regulating the expression of UCP1 [Zhou *et al,* 2003], and both its mRNA and protein are downregulated in the interscapular BAT of rats on cold exposure [Shimizu & Yokotani, 2009]. Conversely, in mice it is upregulated in WAT subjected to 'browning' by prolonged cold exposure [Barneda *et al,* 2013]. Its expression in adipocytes is mediated by PPARγ (Section 1.8.2.3, p 54), directly or indirectly [Puri *et al,* 2008], and is induced by PGC1α (Section 1.8.4.4, p 61) and repressed by RIP140 (Section 1.8.3.6, p 58) [Hallberg *et al,* 2008]. In human adipocytes it is also downregulated by insulin [Ito *et al,* 2010].[16] Mice that are deficient in it are fertile and healthy, but have lean phenotypes, lower WAT mass, smaller white adipocytes, higher core temperature, higher lipolysis in BAT, and higher energy expenditure compared to wild types, and are resistant to high fat diet-induced obesity and T2DM [Zhou *et al,* 2003]. Finally, it has a role in the loss of adipose tissue in cancer cachexia [Laurencikiene *et al,* 2008], and is also expressed in the mammary glands, where it acts as a transcriptional coactivator to control the secretion of milk lipids [Wang *et al,* 2012].

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[13] CIDEC is also known as fat-specific protein (FSP) 27.

[14] CIDEA is a well-established marker of BAT in rodents [Symonds, 2013].

[15] In mice, CIDEC is highly expressed in WAT and moderately expressed in BAT [Zhou *et al,* 2003].

[16] In contrast to CIDEA, CIDEC is upregulated by insulin [Ito *et al,* 2010]. Insulin therefore seems to control the ratio of CIDEA to CIDEC.

1.8.4.3 Deiodinase (DIO) type 2

DIO2 (Figure 1.3, p 26) is an enzyme that converts the thyroid pro-hormone thyroxine (T_4) to the more active hormone T_3. It is a homodimeric membrane-bound selenoprotein (ie it contains the rare amino acid selenocysteine) that resides in the endoplasmic reticulum [Bianco et al, 2002]. It regulates the cellular levels of T_3, which binds to nuclear thyroid hormone receptors, ligand-dependent transcription factors [Cheng et al, 2010] that induce UCP1 (Section 1.8.4.6, p 63) expression. Uniquely, it is post-translationally regulated by an ubiquitin-proteasome mechanism, catalysed by T_4 [Steinsapir et al, 2000]. It is expressed in the brain, pituitary gland and BAT in the rat [Croteau et al, 1996], and in the heart, skeletal muscle, placenta, cochlea, pituitary gland, BAT, fetal brain, and several regions of the adult brain, including the hypothalamus, in humans [Williams & Bassett, 2011]. It is essential for adaptive thermogenesis in BAT, where it is activated by the sympathetic nervous system via noradrenaline release, and its expression increases up to 50-fold in that tissue during cold stress [Bartha et al, 2000; de Jesus et al, 2001; Silva & Larsen, 1983]. Although mice that are deficient in it have normal serum levels of T_3, they develop hypothermia on cold exposure, as a result of impaired thermogenesis. They survive, but only by increased shivering, with consequent severe weight loss [de Jesus et al, 2001; Schneider et al, 2001].

1.8.4.4 Peroxisome proliferator-activated receptor γ coactivator (PGC) 1α

PGC1α (Figure 1.3, p 26) is a transcriptional coactivator that is involved in the regulation of energy metabolism. It was so named because it was first identified as a coactivator of the nuclear receptor PPARγ (Section 1.8.2.3, p 54) in brown adipocytes [Puigserver et al, 1998]. Its close homologue, PGC1β, shares a similar tissue distribution, but is separately regulated and has distinct, if overlapping, functions [Lin et al, 2002; Uldry et al, 2006]. It is expressed mainly in oxidative tissues (ie tissues with a high energy demand) such as the liver, brain, heart, kidney, muscle, pancreas and BAT [Handschin, 2009]. It is induced in a tissue-specific manner by various environmental cues, such as exercise in skeletal muscle and fasting in the liver [Villena, 2015]. In BAT, it is induced by $β_3$-adrenergic stimulation after cold exposure, whereupon it promotes expression of thermogenic genes, such as UCP1 (Section 1.8.4.6, p 63) and DIO2 (Section 1.8.4.3, p 61), by coactivating key transcription factors, including PPARγ, thyroid hormone receptors and ATF2 (Section 1.8.4.1, p 59) [Cao et al, 2004; Puigserver et al, 1998]. It also induces mitochondrial biogenesis by coactivating other transcription factors such as PPARα, nuclear respiratory factors and oestrogen-related receptor α [Finck & Kelly, 2006]. It has been termed the 'master regulator' of both thermogenesis [Sharma et al, 2014] and mitochondrial biogenesis [Ventura-Clapier et al,

2008], and it is essential for BAT-mediated adaptive thermogenesis both *in vivo* and *in vitro* [Handschin, 2009]. However, it is not essential for the differentiation of brown adipocytes, and it does not appear to be required to maintain basal levels of UCP1 and other thermogenic genes at thermoneutrality [Uldry *et al*, 2006].[17] It is only expressed at low levels in WAT, but its forced expression in human and murine white adipocytes induces expression of UCP1 and other BAT-specific genes and promotes mitochondrial biogenesis [Puigserver *et al*, 1998; Tiraby *et al*, 2003]. However, it does not seem to be essential for basal or TZD-induced mitochondrial biogenesis in white adipocytes (a role which may be fulfilled by PGC1β), though it is required for TZD-induced expression of UCP1 and other 'browning' genes [Pardo *et al*, 2011].

1.8.4.5 Prolactin receptor (PRLR)

Prolactin receptor (PRLR) is a cell membrane-bound receptor that is made up of three domains: extracellular, single-pass transmembrane, and intracellular. Alternative splicing or post-translational cleavage give rise to various isoforms, classified as long, intermediate or short, that differ in the length and composition of their intracellular tail. It is activated by dimerisation on binding to a single molecule of ligand, which can be prolactin, growth hormone or placental lactogen. It is found in virtually all adult mammalian tissues, with the highest expression in the liver, mammary gland, adrenal gland and hypothalamus [Bole-Feysot *et al*, 1998; Brandebourg *et al*, 2007]. In sheep, its mRNA and protein are highly expressed in fetal and neonatal BAT [Bispham *et al*, 1999; Symonds *et al*, 1998], and there is a close correlation between its perinatal ontogeny and that of UCP1 (Section 1.8.4.6, p 63), with the expression of both peaking just after birth. Furthermore, treatment of neonatal lambs with prolactin has a thermogenic effect [Pearce *et al*, 2005]. Newborn mice deficient in it have hypertrophic BAT depots that only express low levels of the thermogenic genes UCP1, PGC1α (Section 1.8.4.4, p 61) and β_3-adrenoceptor (ADRB3), and the adipogenic gene PPARγ (Section 1.8.2.3, p 54). Brown adipocyte precursors in which it is knocked out do not differentiate, unless it is reintroduced [Viengchareun *et al*, 2008]. It is also expressed in the WAT of mice and humans, and in the former is upregulated during lactation [Ling & Billig, 2001; Ling *et al*, 2003]. It seems to play a role in adipogenesis [Flint *et al*, 2006; Nanbu-Wakao *et al*, 2000], and its expression increases substantially on differentiation of murine 3T3-L1 adipocytes *in vitro* [Fleenor *et al*, 2006]. It has also been linked with the 'browning' of WAT, but in this case its effect on thermogenesis seems to be inhibitory. Mice deficient in it are

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[17] Although PGC1α and PGC1β are not individually necessary for brown adipocyte differentiation, the presence of at least one of these homologues is required for development of the brown adipocyte phenotype [Uldry *et al*, 2006].

resistant to high fat diet-induced obesity, with reduced fat mass, the appearance of brown-like adipocytes, and increased expression of UCP1, PGC1α, ADRB3 and PRDM16 (Section 1.8.5.3, p 64) in subcutaneous and perirenal WAT depots [Auffret *et al*, 2012].

1.8.4.6 Uncoupling protein (UCP) 1

UCP1 (Figure 1.3, p 26) is the principal mediator of NST. It is only expressed in brown and brite/beige adipocytes,[18] and is used as the principal marker of BAT. It is located in the inner mitochondrial membrane of brown adipocytes, where it is very highly expressed.[19] It uncouples cellular respiration from the production of ATP by enabling protons to dissipate from the intermembrane space to the mitochondrial matrix, bypassing ATP synthase and thus generating heat (Figure 1.2, p 25). It shares considerable homology with several other uncoupling proteins, notably UCP2 and UCP3, but these are only expressed at very low levels and do not mediate adaptive thermogenesis [Brand & Esteves, 2005]. It is regulated by the sympathetic nervous system, acting on environmental cues such as cold exposure, via the binding of noradrenaline to β_3-adrenergic receptors and resultant production of intracellular cyclic adenosine monophosphate (cAMP). It is activated by fatty acids, which are released by lipolysis following β_3-adrenergic stimulation, and it is inhibited by purine nucleotides, such as ATP and GDP [Cannon & Nedergaard, 2004].

1.8.5 Developmental genes

1.8.5.1 Homeobox (HOX) C9

HOXC9 is a member of a large family of similar genes that control the formation of organs and body structures during the early stages of embryonic development, and which encode proteins with a highly-conserved DNA binding region of 60 amino acids (known as the homeodomain). It is one of several developmental genes that are differentially expressed in the various adipose tissue depots of both humans and mice [Gesta *et al*, 2006; Yamamoto *et al*, 2010], although its function in adipose tissue is unknown. It was selected for testing because it had been identified in a number of papers as a marker of brite/beige adipose tissue. This may have arisen because the inguinal and retroperitoneal depots, in which it is particularly highly expressed, had been classified by Waldén *et al* [2012] as brite/beige on the basis that they expressed UCP1 on cold exposure, but not at thermoneutrality. It is also

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[18] There has been one report that UCP1 is expressed in longitudinal smooth muscle of the digestive and reproductive tracts [Nibbelink *et al*, 2001], but this has not been independently confirmed and the finding has been challenged in a subsequent paper [Rousset *et al*, 2003].

[19] UCP1 makes up some 4 % of total protein and 8 % of mitochondrial protein [Rousset *et al*, 2004].

expressed, however, albeit at lower levels, in other WAT depots, and more recent work suggests that, in mice at least, it is not an effective marker of brite/beige adipose tissue [de Jong *et al*, 2015]. Its expression increases in white (but not brown) adipocyte cultures that are subjected to a browning programme (PPARγ activation by rosiglitazone) [Petrovic *et al*, 2010], but does not increase on cold exposure [Waldén *et al*, 2012], so its role in brite/beige cells is unclear. What is clear is that it is expressed at very low levels, or is not expressed at all, in classical BAT depots [Waldén *et al*, 2012; Yamamoto *et al*, 2010] and brown adipocyte precursors [Mohsen-Kanson *et al*, 2014]. Its use as a marker, therefore, is restricted to distinguishing classical BAT from brite/beige adipose tissue and WAT [Cereijo *et al*, 2015].

1.8.5.2 LIM homeobox (LHX) 8

LHX8 is a member of a subfamily of homeobox genes that contain two tandemly-repeated LIM domains and a unique 'LIM-type' homeodomain. The LIM domain is a conserved zinc finger structure that is named after the first three proteins in which it was found (LIN11, ISL1, MEC3) [Bach, 2000; Kitanaka *et al*, 1998]. Although its function in adipose tissue is unknown, it was found to be one of a number of genes that were more highly expressed (> 5-fold) in brown preadipocytes and adipocytes than in white [Seale *et al*, 2007; Timmons *et al*, 2007]. It was subsequently discovered that it was not upregulated when WAT was subjected to a 'browning' programme (ie it was not expressed in brite/beige adipocytes) [Petrovic *et al*, 2010]. This was supported by a further study that found that it was much more highly expressed in (most) murine adipose depots categorised as classical BAT than in those categorised as brite/beige or WAT [Waldén *et al*, 2012]. It has since been used in a number of studies, therefore, as a marker of classical BAT. However, this is at variance with another study which reported that it is a strong marker of subcutaneous, as opposed to visceral, preadipocytes [Macotela *et al*, 2012].

1.8.5.3 PRD1-BF1-RIZ1 homologous domain containing (PRDM) 16

PRDM16 (Figure 1.4, p 31) is expressed in numerous human tissues, including heart, brain, lung, liver, kidney, pancreas and skeletal muscle [Lahortiga *et al*, 2004], and in a wide range of developing murine tissues, including brain, lung, kidney, liver and gastrointestinal tract [Horn *et al*, 2011]. In adipose tissue, it acts as a transcriptional regulator that controls the development of brown adipocytes from myogenic precursors in classical BAT depots, by promoting expression of brown adipocyte-specific genes and suppressing expression of white adipocyte-specific and myogenic genes. Its loss from these precursors causes a loss of brown fat characteristics and promotes muscle differentiation [Seale *et al*, 2008; Seale *et al*, 2007]. It is also expressed, albeit at a lower level than in classical BAT, in subcutaneous,

but not visceral, WAT depots, where it is a determinant of thermogenesis and 'browning'. Futhermore, its ectopic expression in WAT increases expression of the BAT-specific genes UCP1 (Section 1.8.4.6, p 63), CIDEA (Section 1.8.4.2, p 60) and PGC1α (Section 1.8.4.4, p 61) in the inguinal (subcutaneous), but not the epididymal (visceral), depot in mice [Seale *et al*, 2011]. Curiously, however, on cold exposure, which induces the upregulation of most thermogenic genes, it is downregulated in classical BAT depots and remains unchanged in WAT depots, even those susceptible to browning [Waldén *et al*, 2012]. Finally, its adipocyte-specific deletion in mature adipocytes (as opposed to precursors) in mice caused minimal effects on the thermogenic programme in classical BAT, but notably inhibited brite/beige adipocyte function in subcutaneous WAT following cold exposure or treatment with a $β_3$-adrenergic agonist. Animals with this deletion developed obesity and severe insulin resistance on a high-fat diet, and showed an altered fat distribution pattern, with a marked increase in subcutaneous adiposity. Furthermore, their subcutaneous fat acquired many of the properties of visceral fat, including a reduced thermogenic capability and increased inflammatory gene expression and macrophage infiltration [Cohen *et al*, 2014].

1.8.5.4 Short stature homeobox (SHOX) 2

SHOX2 is a member of the homeobox family of developmental genes, and is involved in craniofacial, brain, heart and limb development [Blaschke *et al*, 1998]. In common with a number of other developmental genes, it is differentially expressed in the various adipose tissue depots, being highly expressed in subcutaneous WAT, but only minimally expressed in BAT and visceral WAT [Gesta *et al*, 2006; Lee *et al*, 2013; Waldén *et al*, 2012; Yamamoto *et al*, 2010]. Its mRNA levels do not change in either humans or mice during adipogenesis. Its expression in subcutaneous adipose tissue is higher in humans with visceral adiposity than in those with subcutaneous adiposity or lean individuals. Mice deficient in it do not survive beyond mid-gestation due to cardiovascular defects, but it can be knocked down in adipose tissue selectively without producing any obvious abnormalities. In male mice, its adipose tissue-specific disruption protects against high fat diet-induced obesity, with a reduction in hypertrophy (ie adipocyte size), particularly in the subcutaneous depot. There is also an increase in the rate of catecholamine-induced lipolysis, with a corresponding increase in expression of ADRB3. In contrast, its overexpression leads to a reduction in lipolysis and ADRB3 expression. In summary, therefore, it may play a role in determining the function, distribution and phenotype of adipose tissue in both humans and mice, possibly through regulation of ADRB3 expression [Lee *et al*, 2013]. Finally, it was selected for testing because it had been considered to be a marker of brite/beige adipose tissue, but this seems to stem from a misinterpretation of a paper by Waldén *et al* [2012]. In reality, it seems to be simply

a marker of inguinal adipose tissue, the largest subcutaneous depot in mice, which the authors classified as brite/beige adipose tissue rather than WAT because it expressed UCP1 on cold exposure.

1.8.6 Concluding remarks

Although expression of many of the important genes described above (eg ADIPOQ, INSR, LEP, NR3C1, PGC1α, PPARγ and UCP1) in ovine adipose tissue has been examined before in a number of different studies, limited work has been done on the expression of other genes (eg CIDEA and developmental genes), and the expression of some genes (eg ATF2, C/EBPα and GPR120) does not appear to have been studied at all. Furthermore, it is reiterated that no previous work has been done on gene expression in the sternal adipose tissue of sheep.

Table 1.2 Summary of functional role(s) of genes of interest. A more detailed description of the functional role(s) of each of the genes listed, together with the relevant citations, is provided in the section of the main text referenced in the 'Category/ref' column. The full name of each of the genes listed is shown in the referenced sections and in the List of Abbreviations and Acronyms (p 15).

Gene	Category/ref	Functional role(s)
ADIPOQ	Metabolism 1.8.3.1, p 56	Adiponectin, an adipokine and the most abundant peptide secreted by adipocytes. Increases insulin sensitivity and plays an important role in controlling energy homeostasis through regulation of glucose and lipid metabolism in liver and muscle. Its expression is negatively correlated with BMI, though that relationship is reversed in the immediate postnatal period. Has antiatherogenic and antiinflammatory properties, and may inhibit expression of UCP1. Regulated by C/EBPα, PPARγ and SREBP1c.
ATF2	Thermogenesis 1.8.4.1, p 59	Multi-functional transcription factor that plays a role in thermogenesis in BAT by promoting expression of UCP1 and PGC1α. Also expressed in WAT and liver, where it influences glucose and lipid metabolism. Plays a role in early adipogenesis by regulating expression of PPARγ. Highly expressed in macrophages. Promotes expression of inflammatory genes (eg TNFα).
C/EBPα	Adipogenesis 1.8.2.1, p 53	One of the two main regulators of late-stage adipogenesis in WAT, where it induces and maintains PPARγ expression. Not required for embryonic or BAT adipogenesis. Critical in establishing insulin sensitivity. Promotes expression of leptin and adipsin in mature adipocytes, and regulates genes involved in glycogen synthesis and gluconeogenesis in the liver.
CIDEA	Thermogenesis 1.8.4.2, p 60	Mediates enlargement of lipid droplets in adipose tissue, and plays a key role in energy metabolism. Highly expressed in BAT in mice and used as a marker of that tissue, though paradoxically it seems to inhibit thermogenesis by suppressing UCP1 expression. Not expressed in WAT in mice, but highly expressed in human WAT. Plays a role in the loss of adipose tissue in cancer cachexia. Also expressed in the mammary glands, where it has a role in controlling secretion of milk lipids. Regulated by PPARγ.
DIO2	Thermogenesis 1.8.4.3, p 61	Enzyme that converts the thyroid hormone T_4 to the more active T_3. Expressed in BAT in humans and rats, and essential for NST in that tissue. Also expressed in the brain and pituitary gland of rats and humans, and in the skeletal muscle, heart and placenta of humans.

Gene	Category/ref	Functional role(s)
FABP4	Metabolism 1.8.3.2, p 56	Intracellular chaperone that reversibly binds hydrophobic ligands such as long-chain fatty acids and eicosanoids, distributing them for storage, enzyme activation and signalling. Mainly expressed in adipose tissue, where it occurs at very high levels and is used as a marker of adipocyte differentiation, and macrophages. Induced by PPARγ and C/EBPα. May also be a circulating protein and act as an adipokine, and may negatively regulate adipogenesis by triggering proteasomal degradation of PPARγ.
GPR120	Metabolism 1.8.3.3, p 57	Cell membrane-bound receptor for long-chain fatty acids that may mediate fatty acid regulation of energy homeostasis. Especially selective for ω-3 fatty acids and mediates their antiinflammatory and insulin-sensitising effects. Its activation increases glucose transport in adipocytes and induces secretion of incretins in the intestine. Expressed in taste buds, where it may mediate taste preference for fatty acids. May also play a role in adipogenesis and be a factor in the 'browning' of WAT.
HOXC9	Development 1.8.5.1, p 63	Homeobox gene that is differentially expressed in the various adipose depots of humans and mice. Its function in adipose tissue is unknown. Originally suggested as a marker of brite/beige adipose tissue, but not an effective marker of that tissue as also expressed in WAT. Expression increases in WAT cultures subjected to browning by PPARγ activation, but not on cold exposure. Expressed at very low levels, if at all, in BAT.
INSR	Metabolism 1.8.3.4, p 57	Insulin receptor, a cell membrane-bound receptor. Binding of its ligands, usually insulin or IGFs, activates signalling pathways that regulate energy metabolism (eg by facilitating glucose uptake) or control cell growth and differentiation, including adipogenesis, where its substrates play a crucial role in the upregulation of PPARγ and C/EBPα.
LEP	Metabolism 1.8.3.5, p 58	Leptin, an adipokine mainly secreted by white adipocytes. Plays a key role in energy homeostasis, especially control of WAT mass, which correlates to its mRNA, protein and plasma levels. Signals the body's nutritional state to various physiological systems. First expressed in the late stages of adipogenesis, induced by PPARγ and C/EBPα. Plays a role in reproduction and development, and in regulating cardiovascular and immune systems.
LHX8	Development 1.8.5.2, p 64	Homeobox gene of unknown function in adipose tissue that is much more highly expressed in brown adipocytes than in white. Not expressed in brite/beige adipose tissue, so used as a marker of classical BAT.
NR3C1	Adipogenesis 1.8.2.2, p 54	Glucocorticoid receptor, a ligand-activated transcription factor that mediates numerous physiological processes, including glucose and lipid metabolism and the inflammatory response. Also essential for late-stage adipogenesis, where it indirectly promotes expression of PPARγ. Inhibits expression and function of UCP1 in brown adipocytes.
PGC1α	Thermogenesis 1.8.4.4, p 61	Transcriptional coactivator involved in regulation of energy metabolism. Mainly expressed in oxidative tissues such as liver, brain, heart, kidney, muscle and BAT. In BAT, it promotes expression of thermogenic genes such as UCP1 and DIO2 by coactivating key transcription factors such as PPARγ, thyroid hormone receptors and ATF2. Also induces mitochondrial biogenesis, and is essential for BAT-mediated adaptive thermogenesis *in vivo* and *in vitro*. Only expressed at low levels in WAT.
PPARγ	Adipogenesis 1.8.2.3, p 54	Ligand-activated nuclear receptor and transcription factor that is the 'master regulator' of adipogenesis and is an important regulator of whole body glucose and lipid metabolism. Promotes FFA uptake and triglyceride storage in WAT, induces expression of adiponectin, and reduces inflammation in adipose tissue. Binding of TZDs and its other ligands strongly stimulates UCP1 expression in BAT and 'browning' of WAT. Also promotes mitochondrial biogenesis and thermogenesis on binding to PGC1α.

Gene	Category/ref	Functional role(s)
PRDM16	Development 1.8.5.3, p 64	Transcriptional regulator expressed in numerous human and developing murine tissues. In classical BAT depots, it controls development of brown adipocytes from myogenic precursors, by promoting expressing of brown adipocyte-specific genes and suppressing expression of myogenic and white adipocyte-specific genes. Expressed at lower levels in subcutaneous WAT depots, where it regulates thermogenesis and 'browning' of WAT.
PRLR	Thermogenesis 1.8.4.5, p 62	Cell membrane-bound receptor found in most mammalian tissues, but highly expressed in the liver, mammary and adrenal glands, and hypo-thalamus. In sheep, its mRNA and protein are highly expressed in fetal and neonatal BAT, and there is a close correlation between its perinatal ontogeny and that of UCP1. Treatment of neonatal lambs with its ligand prolactin has a thermogenic effect. May also play a role in adipogenesis.
RIP140	Metabolism 1.8.3.6, p 58	Ligand-dependent corepressor of nuclear receptors, such as PPARγ, that are important regulators of energy homeostasis. Highly expressed in liver, muscle and adipose tissue. Suppresses catabolic processes such as fatty acid oxidation and glycolysis. Inhibits expression of UCP1, CIDEA and other thermogenic genes in WAT, thus preventing 'browning' of the tissue. Also plays a role in the cytoplasm, where it suppresses glucose uptake and adiponectin secretion. Widely expressed and controls various physiological processes, and can act as a transcriptional coactivator.
SHOX2	Development 1.8.5.4, p 65	Homeobox gene involved in craniofacial, brain, heart and limb development. Highly expressed in subcutaneous WAT, but only expressed at very low levels in visceral WAT and BAT. May play a role in determining the function, distribution and phenotype of adipose tissue in humans and mice, possibly through regulation of ADRB3 expression. Once considered to be a marker of brite/beige adipose tissue.
SREBF1	Adipogenesis 1.8.2.4, p 55	Encodes SREBP1a and SREBP1c. SREBP1c, the main isoform in adipose tissue and liver, regulates lipogenesis and is induced by insulin. Has long been linked to adipogenesis, although its role is unclear. May enhance the effect of PPARγ and promote the production of PPARγ ligands *in vitro*, but may not be necessary for adipogenesis *in vivo*. SREBP1a, a more powerful factor than SREBP1c, is mainly regulated by cholesterol levels. Has been recently linked to preadipocyte proliferation and adipogenesis *in vitro*.
UCP1	Thermogenesis 1.8.4.6, p 63	Inner mitochondrial membrane-bound protein that is only expressed in brown and brite/beige adipocytes. Principal mediator of NST. Uncouples cellular respiration from the production of ATP, thereby generating heat. Regulated by sympathetic nervous system, acting on environmental cues such as cold exposure, through binding of noradrenalin to β_3-adrenergic receptors. Activated by fatty acids and inhibited by purine nucleotides.

2 Materials and methods

2.1 Introduction

The work was carried out in two different studies, hereafter labelled A and B. Study A mainly examined the effect of fat supplements given to lactating ewes on gene expression in the sternal adipose tissue of their 7 and 28 day-old suckling lambs (Chapter 4). A subset of lambs from Study A was also used to examine the ontogeny of sternal adipose tissue in the first month after birth (Chapter 3). Study B looked at the effect of changes in late gestational and postnatal nutrition on gene expression in the sternal and subcutaneous adipose tissue of 6 month-old lambs (Chapter 5). This chapter details the general methodology used, which, except where otherwise specified, was the same for both studies. The labelling of the studies, and the order in which the results chapters have been set out in this thesis, is based on the timing and duration of interventions. However, the work for Study B was carried out before the work for Study A, so there are certain refinements of methodology in the latter study. The supplier for primers and laboratory materials in the UK, except where otherwise indicated, was Sigma-Aldrich, Gillingham, Dorset, UK. The full details of all supplier names and addresses are shown in Appendix A, p 279.

2.2 Animal husbandry and interventions

The detailed procedures for animal husbandry and interventions for Studies A and B are described in Section 4.3.1, p 149, and Section 5.3, p 196, respectively. The animal work for Study B was carried out in Denmark by colleagues from the University of Copenhagen.

2.3 Plasma analysis

Concentrations of leptin [Delavaud *et al*, 2000] and IGF1 [Lalman *et al*, 2000; Morrison *et al*, 2002] in the plasma of Study A lambs were determined by Prof Duane Keisler's group at the University of Missouri using double-antibody radioimmunoassays, as previously described.

2.4 Gene expression

2.4.1 Introduction

Genes are 'transcribed' into messenger RNA (mRNA), which in turn is 'translated' into protein. The amount of a gene's mRNA in a cell or tissue is determined by the rate of transcription (or 'expression') of the gene and the rates of translation and/or degradation of its mRNA. The relative quantities of a gene's mRNA in different samples or tissues therefore give an indication of its relative rates of expression in those samples or tissues.

The method used to determine the amount of mRNA in a sample was a combination of reverse transcription (RT) and the polymerase chain reaction (PCR), a process sometimes referred to as RT-PCR.[20] RT is a technique used to convert mRNA into DNA, and PCR is a process which exponentially increases the amount of a specific DNA in a sample, thereby enabling its detection and quantification. For the latter step, an enhancement of the basic PCR method, termed quantitative real-time PCR (qPCR), was used.

2.4.2 Basic principles of PCR, RT and qPCR

2.4.2.1 Polymerase chain reaction (PCR)

PCR is an *in vitro* technique to amplify a short section (termed an 'amplicon') of a specific DNA (ie a gene) from a mixture containing numerous different DNAs [Bustin, 2012]. It allows very small amounts of DNA, as low as single digit molecules, to be quantified. It was developed in the mid-1980s by Kary Mullis[21] from earlier work by Kleppe *et al* [1971], and makes use of a cell's own DNA-replication machinery [Mullis *et al*, 1986; Mullis & Faloona, 1987; Saiki *et al*, 1985]. The technique utilises a pair of 'primers', small sequences of single-stranded DNA comprising some 20 base pairs (bp), which are complementary to a known DNA sequence of the gene of interest. One ('forward') primer is complementary to the 3'–5' strand of DNA, and the other ('reverse') primer is complementary to the 5'–3' strand. They are usually designed so that they define an amplicon of 70–250 bp. They are combined in a reaction mixture with DNA, deoxynucleoside triphosphates (dNTPs), a DNA polymerase and a buffer (containing ions such as K^+ and Mg^{2+}). Reagents other than the template DNA are usually made up as a reaction 'master mix' to minimise inter-sample variation.

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[20] Confusingly, the abbreviation RT is used in the literature to refer to both 'reverse transcription' and 'real time' when applied to PCR. In this thesis it is used solely for reverse transcription, as recommended in The MIQE Guidelines [Bustin *et al*, 2009].

[21] Kary Mullis shared the 1993 Nobel Prize in Chemistry for the development of PCR.
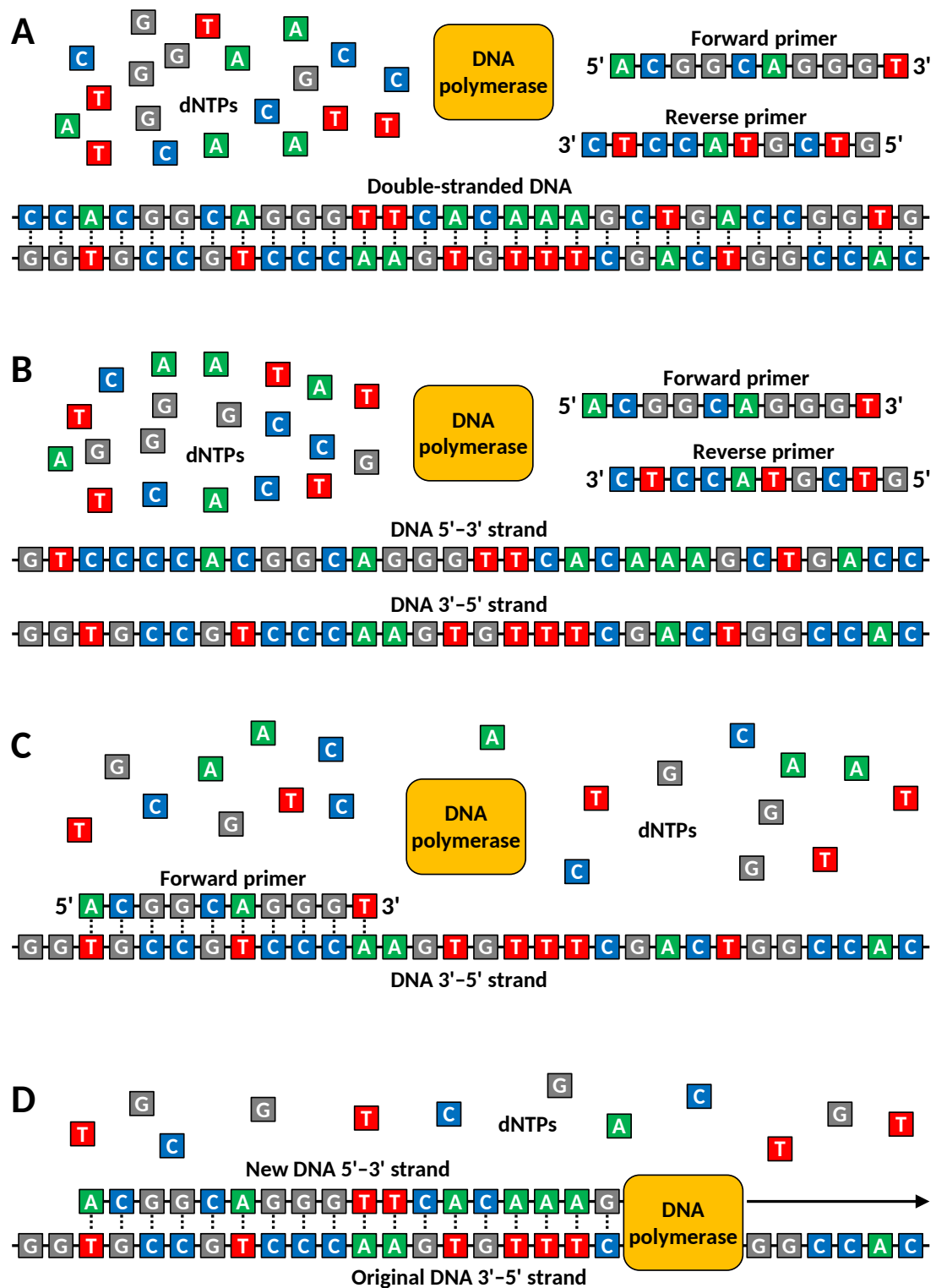
Figure 2.1 Primer extension by a DNA polymerase. A. Components of the reaction mixture: primers, DNA, deoxynucleoside triphosphates (dNTPs) and DNA polymerase. **B.** Melting stage (95 °C): DNA is denatured and the two strands separate. **C.** Annealing stage (60 °C): the forward primer binds to its complementary sequence on the 3'–5' strand; the reverse primer binds to its complementary sequence on the 5'–3' strand (not shown). **D.** Extension stage (60–72 °C): DNA polymerase synthesises a new 5'–3' strand of DNA on the original 3'–5' strand by incorporating dNTPs in accordance with the normal Watson-Crick base-pairing rules; a similar process occurs on the original 5'–3' strand (not shown). Note that in reality primers would normally have around 20 base pairs. Figure adapted from McPherson & Møller [2006].

The amplification process involves 3 temperature-dependent stages (Figure 2.1, p 71):

1. melting;

2. annealing;

3. extension.

The mixture is initially heated to about 95 °C, at which point the two strands of template DNA will separate or 'melt'. The temperature is then rapidly lowered to around 60 °C, at which point the primers will bind or 'anneal' to the resulting single strands of DNA.[22] The short 'primed' sequences of double-stranded DNA provide a starting point for the DNA polymerase to 'extend' or complete the second strand, by incorporating the appropriate dNTP at each base to conform with normal Watson-Crick base-pairing rules. The optimum temperature for extension is around 72 °C, but some protocols retain the temperature at 60 °C for this stage, as the DNA polymerase has sufficient activity (~50 %) at the annealing temperature to complete the extension process [Bustin, 2012]. When the DNA has been sufficiently extended, the temperature is rapidly increased again, and the full sequence of melting, annealing and extension is repeated for some 25–40 cycles (Figure 2.2, below).
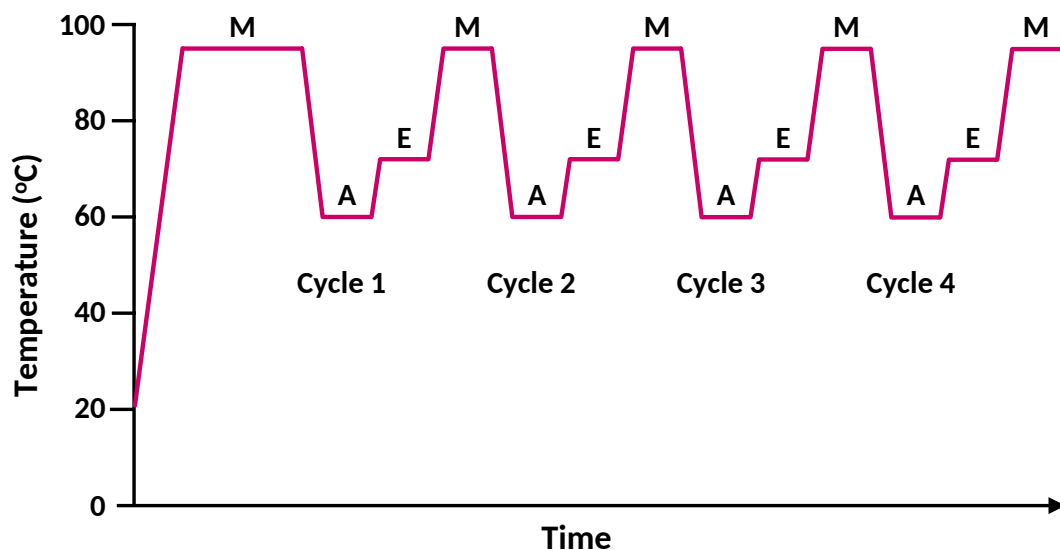
Figure 2.2 Thermal cycling during the polymerase chain reaction (PCR). The PCR reaction mixture is subjected to a repeated sequence of stages at different temperatures: a melting stage (**M**), where the two DNA strands denature and separate; an annealing stage (**A**), where primers bind to their complementary sequences on single strands of DNA; and an extension stage (**E**), where a DNA polymerase synthesises a second strand of DNA from deoxynucleoside triphosphates (dNTPs) according to the normal Watson-Crick base-pairing rules. The sequence is repeated for 25–40 cycles, after which the mixture is cooled to 4 °C. The initial melting stage is extended to ensure that the template DNA is fully denatured and to activate a 'hot-start' DNA polymerase. Figure adapted from McPherson & Møller [2006].

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[22] At this temperature (60 °C) the individual strands of template DNA are too long and complex to bind together again in the short time available [McPherson & Møller, 2006].

In the early days of PCR, the process was largely manual, with temperature changes being effected by the use of water baths, and the DNA polymerase being replenished at the start of each extension stage, as it denatured at high temperatures [McPherson & Møller, 2006]. Nowadays, the thermally stable *Taq* polymerase (from the thermophilic bacterium, *Thermus aquaticus*) is used, in combination with thermal cyclers, to automate the process [Saiki *et al*, 1988]. The *Taq* polymerase is usually modified so that it has so-called 'hot-start' functionality. This stops the polymerase from working until it has been activated at a high temperature over a defined period, and prevents 'false priming' while the reaction mixture is being brought up to the minimum cycle temperature. False priming occurs mainly at lower temperatures where primers can more easily bind to incorrect sequences [Shipley, 2006]. Thermal cyclers are programmable incubators that automate and regulate the rapid change of temperatures required in a PCR reaction. They usually incorporate a heated block that can hold individual tubes and/or a microtitre plate [Bustin, 2012].

In addition to amplicons of defined length, a number of intermediate DNA fragments of indeterminate length are generated during amplification that predominate in the first few cycles. Indeed, the first discrete amplicons do not appear until the end of the third cycle (Figure 2.3, p 74). However, the number of amplicons starts to grow exponentially from cycle 4 onwards, and as the number of cycles increases, the intermediate products become increasingly irrelevant.

If the amplification process were perfectly efficient, the number of copies of DNA would double after each cycle (Figure 2.3, p 74). After 20 cycles, therefore, a single molecule of template DNA would have produced over a million amplicons (Table 2.1, p 75). In practice, however, the kinetics of the reaction are such that it is neither perfectly efficient nor continuously exponential over its course. Contaminants within the reaction mixture, for example haemoglobin or reagents from reverse transcription, can inhibit DNA polymerase activity, thereby reducing efficiency. Efficiency can also be affected by small temperature differences on the heated block of plate-based thermocyclers, and by inaccurate pipetting (ie well-to-well variations in the amount of reaction master mix). For a perfectly efficient reaction, however, the amount of product at the end of each cycle would be as shown in Equation 2.1:

$$N_A = N_0 \cdot 2^n \qquad\qquad (2.1)$$

where:

N_A = number of amplicons;
N_0 = initial number of template DNA molecules;
n = number of amplification cycles.

DNA product	Cycle 1 No	Cycle 2 No	Cycle 3 No	Cycle 4 No
	1	1	1	1
	1	1	1	1
	0	1	2	3
	0	1	2	3
Ⓐ	0	0	2	8
Total	**2**	**4**	**8**	**16**
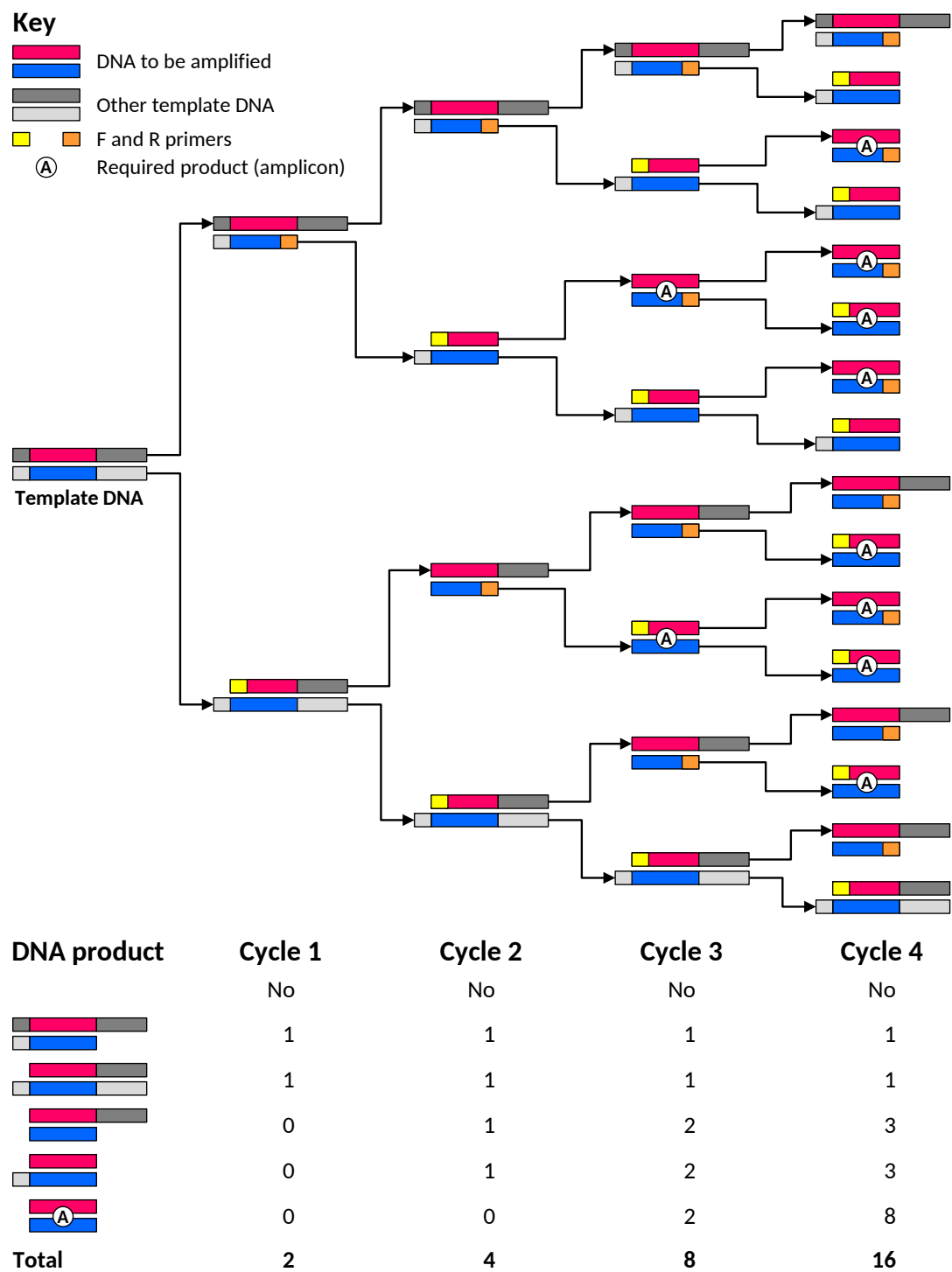
Figure 2.3 Amplification of DNA by PCR. The top half of the figure shows amplification of DNA by PCR for 4 cycles of melting, annealing and extension. If the process were perfectly efficient, the amount of DNA would double after each cycle. Some intermediate products of indeterminate length are formed, which predominate in the early cycles. The first product of defined length, the required product (amplicon), appears after Cycle 3. From Cycle 4 onwards, the amount of required product increases exponentially, and the effect of intermediate products becomes increasingly immaterial (Table 2.1, p 75). The table at the bottom of the figure shows the number of products by type at the end of each cycle. F is forward, R is reverse.

Table 2.1 PCR amplification of a section of a single DNA molecule over 20 cycles of melting, annealing and extension. Figures are shown for both a double-stranded DNA template and a single-stranded complementary DNA (cDNA) template. In the latter case, the first cycle is not an amplification, it adds a complementary strand to the template to form a (partially) double-stranded molecule. If the process were perfectly efficient, the amount of DNA would double after each cycle. Some intermediate products of indeterminate length (shown under 'Other DNA') are formed, which predominate in the early cycles (Figure 2.3, p 74; Figure 2.6, p 79). The first product of defined length, the required product (amplicon), appears after Cycle 3. From Cycle 4 onwards, the amount of required product increases exponentially, and the effect of intermediate products becomes increasingly immaterial.

Cycle	Double-stranded DNA template			Single-stranded cDNA template		
	Amplicons	Other DNA	Total copies	Amplicons	Other DNA	Total copies
	No	No	No	No	No	No
0	0	1	1	0	½	½
1	0	2	2	0	1	1
2	0	4	4	0	2	2
3	2	6	8	1	3	4
4	8	8	16	4	4	8
5	22	10	32	11	5	16
6	52	12	64	26	6	32
7	114	14	128	57	7	64
8	240	16	256	120	8	128
9	494	18	512	247	9	256
10	1 004	20	1 024	502	10	512
11	2 026	22	2 048	1 013	11	1 024
12	4 072	24	4 096	2 036	12	2 048
13	8 166	26	8 192	4 083	13	4 096
14	16 356	28	16 384	8 178	14	8 192
15	32 738	30	32 768	16 369	15	16 384
16	65 504	32	65 536	32 752	16	32 768
17	131 038	34	131 072	65 519	17	65 536
18	262 108	36	262 144	131 054	18	131 072
19	524 250	38	524 288	262 125	19	262 144
20	1 048 536	40	1 048 576	524 268	20	524 288

In practice, it is necessary to correct for imperfect efficiency, as shown in Equation 2.2:

$$N_A = N_0(1 + E)^n \qquad\qquad (2.2)$$

where:

E = amplification efficiency (being 1 for 100 % efficiency).

With well-designed primers, efficiencies of over 95 % are achievable.

As the number of target amplicons increases beyond a certain level, amplification stops being exponential, becoming linear for a period, before declining further and eventually ceasing completely (Figure 2.4, p 76). The reasons for this include diminishing reagents,
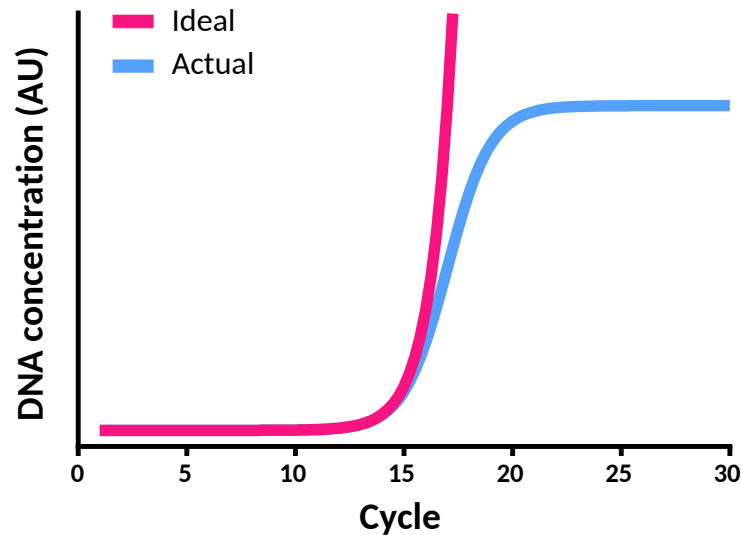
Figure 2.4 Ideal versus actual amplification by PCR. In the ideal scenario, amplification is exponential throughout the reaction (though it may not be detectable for the first few cycles). In the actual situation, amplification is initially exponential, but reaches a point where it starts to decline, firstly to a linear rate, and then still further until it ceases completely. Figure adapted from Shipley [2006].

inhibition of DNA polymerase at high cDNA concentrations, and re-annealing of amplicon strands at high concentrations and temperatures.

The fact that 'classical' PCR reactions reach a stage after which no further amplification occurs makes them a poor tool for the quantification of DNA, as all samples will eventually produce a similar amount of product, irrespective of their initial amounts. Classical PCR is therefore more a qualitative than a quantitative assay [Bustin, 2006]. When the reaction is complete, the amplified DNA is usually assessed by electrophoresis on an agarose or poly-acrylamide gel after staining with ethidium bromide, a fluorescent dye that intercalates between DNA base pairs. Bands of DNA on the gel can then be visualised under ultraviolet light [Newton & Graham, 1997]. The relative expression of genes in different samples can be crudely estimated by band densitometry, but product yield can be substantially affected by minimal variations in the reagent quantities and thermal cycling conditions, and by the reaction kinetics [Bustin, 2006].

2.4.2.2 Reverse transcription (RT)

RT is a technique used by RNA retroviruses to convert their genomic RNA into DNA for incorporation into a host's genome. It is effected by the enzyme 'reverse transcriptase' in a 2-stage process (Figure 2.5, p 77) [Phillips, 2006]:
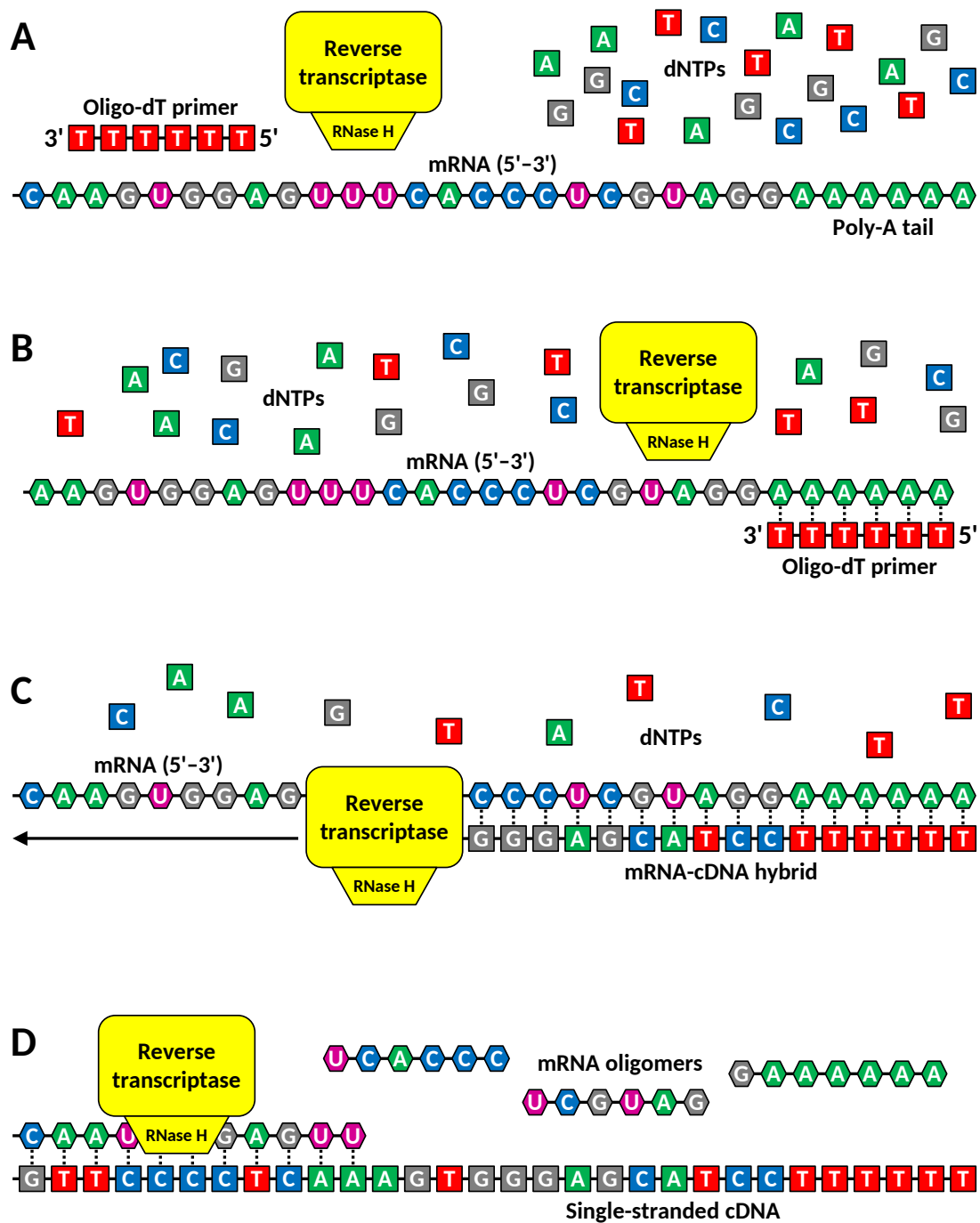
Figure 2.5 Reverse transcription of mRNA to cDNA. A. Components of the reaction mixture: oligo-dT primers, mRNA, dNTPs and reverse transcriptase. **B**. The oligo-dT primers bind to the poly-A tail of the mRNA; the poly-A tail is a long sequence of adenine nucleotides added to mRNA during RNA processing to stabilise the molecule. **C**. RNA polymerase synthesises a strand of complementary DNA (cDNA) on the mRNA template by incorporating dNTPs in accordance with the normal Watson-Crick base-pairing rules, creating an mRNA-cDNA hybrid. **D**. The 'RNase H' domain of the RNA polymerase has endonuclease activity; it breaks down the mRNA strand, leaving single-stranded cDNA and mRNA oligomers. Note that in reality the poly-A tail would be much longer than that shown in the figure. Random hexamers could have been used instead of (or in addition to) oligo-dTs to prime the reaction.

1. synthesis of a DNA strand using the viral RNA as a template to create an RNA-DNA hybrid;

2. degradation of the RNA strand of the RNA-DNA hybrid to leave a single strand of DNA.

Degradation of the RNA strand is effected by a specific domain of the RNA polymerase, termed the 'RNase H' domain, which has endonuclease capability. The resulting single-stranded DNA is known as complementary DNA (cDNA). Reverse transcriptases from either the avian myeloblastosis virus or Moloney murine leukaemia virus (MMLV) are normally used for the reverse transcription of mRNA [McPherson & Møller, 2006]. Total mRNA is extracted from a tissue sample, its concentration is measured, and any residual genomic DNA (gDNA) is removed. cDNA is then prepared from it using a reverse transcriptase, random hexamers and/or oligo-dTs (short sequences of deoxythymine nucleotides which bind to the poly-A tail of mRNA molecules), and dNTPs. In practice these reagents are often combined in a manufacturer's kit. The random hexamers and/or oligo-dTs act as primers for DNA synthesis (Figure 2.5, p 77).

Once cDNA has been produced from mRNA, it can be amplified by PCR like any other DNA. However, the number of amplicon copies after each cycle is half what it would be with double-stranded DNA, as the first cycle is not an amplification: it adds a second strand to the single-stranded template to form a (partially) double-stranded molecule (Figure 2.6, p 79; Table 2.1, p 75).

RT is the least efficient and most variable step in RT-PCR. Reaction efficiency is dependent on both the RNA concentration and on the amount of RT inhibitors in any sample. An efficiency of around 40 % would be typical for the MMLV reverse transcriptase at normal concentrations (ie 10 molecules of RNA would produce 4 molecules of cDNA) [Ståhlberg *et al*, 2004a; Ståhlberg *et al*, 2004b].

2.4.2.3 Real-time quantitative PCR (qPCR)

The key breakthrough which allowed accurate quantification of DNA amplified by PCR was the development by Applied Biosystems of a thermal cycler (the 7700) which contained a built-in fluorimeter [Heid *et al*, 1996]. Such 'qPCR machines' allow the progress of a PCR reaction to be monitored and recorded in real time. Essentially, the amount of DNA present at any point in the process is linked to fluorescence, which is measured and recorded during each cycle, allowing quantification of the accumulating product. Fluorescence is a phenomenon whereby a dye molecule is excited by, and absorbs, light at one wavelength and subsequently re-emits light at a slightly higher wavelength [Bustin, 2012]. In addition
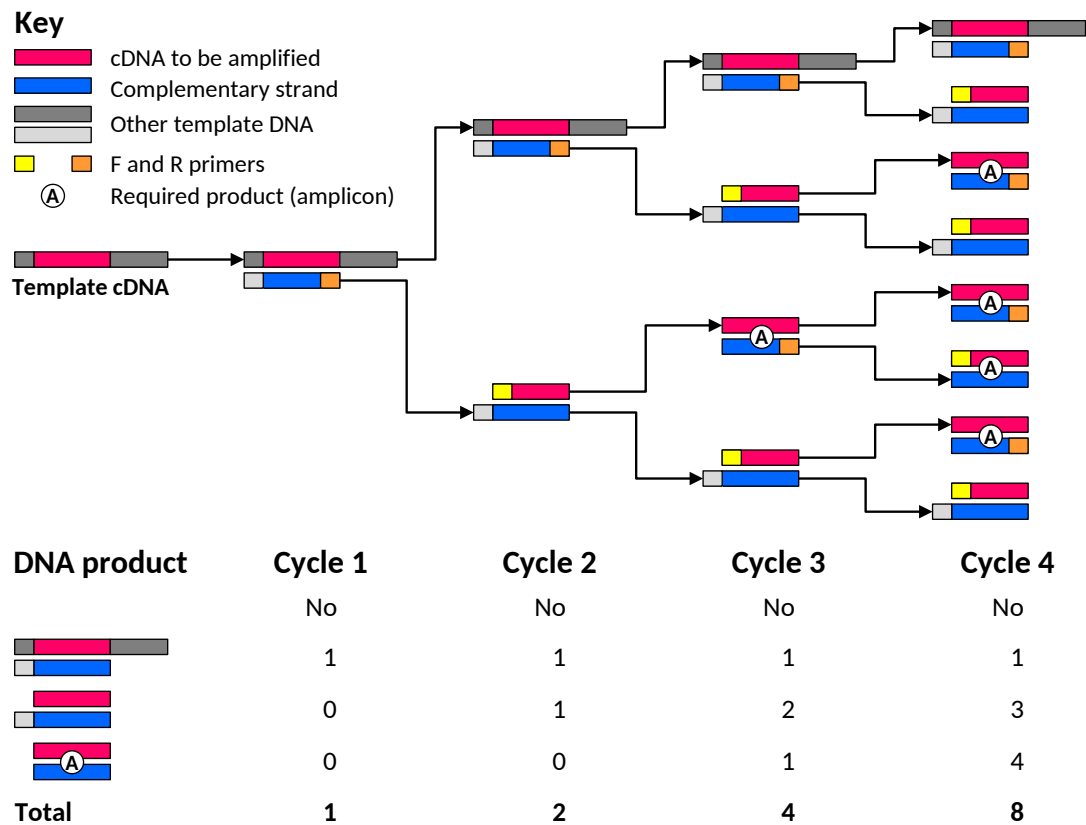
Figure 2.6 Amplification of single-stranded cDNA by PCR. The top half of the figure shows amplification of a single-stranded cDNA template by PCR for 4 cycles of melting, annealing and extension. Note that the first cycle is not an amplification: it adds a complementary strand to the template to form a (partially) double-stranded molecule. If the process were perfectly efficient, the amount of DNA would double after each cycle. Some intermediate products of indeterminate length are formed, which predominate in the early cycles. The first product of defined length, the required product (amplicon), appears after Cycle 3. From Cycle 4 onwards, the amount of required product increases exponentially, and the effect of intermediate products becomes increasingly immaterial (Table 2.1, p 75). The table at the bottom of the figure shows the number of products by type at the end of each cycle. F is forward, R is reverse.

to the normal PCR reagents, a qPCR reaction mixture also contains a fluorescent reference dye, usually ROX, which is used to correct for well-to-well differences in reagent volume, and a fluorescent reporter, which can take one of two main forms [McPherson & Møller, 2006]:

- a fluorescent 'probe';

- a fluorescent DNA-binding dye.

The probe method was not used in this study, so is only mentioned briefly. A probe is a small sequence of nucleotides which works exactly like a primer, except that it is designed to anneal to a complementary sequence between the primer-binding sites of an amplicon. Importantly, therefore, it is gene-specific. It is linked to a both a fluorescent reporter dye and a quencher molecule. The quencher absorbs fluorescence from the reporter when the

two molecules are in close proximity, but the action of *Taq* polymerase cleaves the probe and releases the reporter and quencher. The two molecules then move apart, facilitating detection of the fluorescence [Holland *et al*, 1991; McPherson & Møller, 2006].

The most common fluorescent reporter dye used for the second method, and the one used throughout this study, is SYBR Green I, which binds to the minor groove of double-stranded DNA. It absorbs blue light at around 497 nm, and re-emits green light at around 520 nm [Bustin, 2012]. It is only weakly fluorescent when unbound in solution, but when bound to double-stranded DNA it undergoes a conformational change that increases its fluorescence more than 1 000-fold [Bustin, 2012; Dragan *et al*, 2012]. It does not bind to single-stranded nucleic acids, so the amount of fluorescence detected (after subtraction of any background level) is proportional to the amount of DNA in the reaction mixture. However, the absolute level of fluorescence generated is correlated to the length of the specific amplicon (ie longer DNA sequences can bind more SYBR Green molecules).

SYBR Green will bind to any DNA present in the reaction mixture so, unlike the probe method, it is not gene-specific. It is therefore important to eliminate gDNA and any other contamination, and to design primers which minimise false priming and the formation of 'primer-dimers'. Primer-dimers are artefacts formed by the self-priming of two primers (either a pair of the same primers, or one forward and one reverse primer) followed by DNA polymerase extension (Figure 2.7, p 81). The main cause of dimerisation is sequence complementarity at the 3'-ends [McPherson & Møller, 2006].

In spite of its non-specificity, the advantages of the SYBR Green method are that it can be used with any gene, it is easy to use, it avoids the time factor associated with probe design and testing, and it is cheaper than the probe method [McPherson & Møller, 2006; Morrison *et al*, 1998]. Furthermore, the presence of secondary products can usually be detected by melt curve analysis, as explained below.

At the end of a SYBR Green qPCR reaction, each tube or well of a microtitre plate is heated. As the temperature rises, the DNA will start to denature and the fluorescence will decrease. The melting temperature T_m is the temperature at which a negative first derivative curve of fluorescence over temperature ($-dF/dT$) reaches a peak [Ponchel, 2006]. If there is a single product, there should be a sharp single peak. If there is more than one product, there will be multiple peaks or a single broad peak (Figure 2.8, p 82). It is usual in a qPCR experiment to test for the presence of contaminants by running a number of non-template controls (NTCs) that contain all components of the reaction mixture except the DNA template. Evidence of primer-dimers is often seen in the melt curves of NTCs, but any hint of them in the melt curves of test samples (ie a smaller secondary peak at a lower temperature) compromises quantification of the desired product.

A

B

C
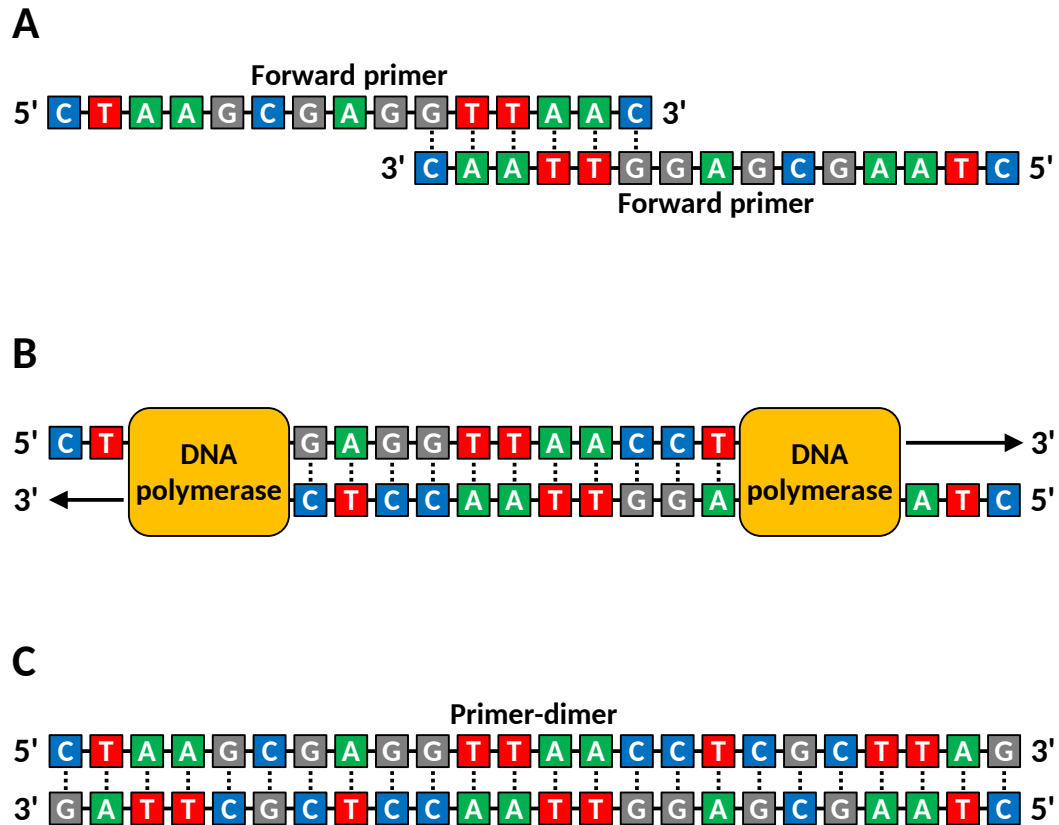
Figure 2.7 Formation of a primer-dimer during PCR. A. Two primers with sequence complementarity (usually at the 3'-ends) anneal together. These could be 2 forward primers (as shown), 2 reverse primers, or 1 forward and 1 reverse primer. **B**. DNA polymerase synthesises new strands of DNA by incorporating dNTPs in accordance with the normal Watson-Crick base-pairing rules. **C**. The finished product, a double-stranded primer-dimer. Primer-dimers formed from 2 forward or 2 reverse primers are termed 'self-dimers'. Those formed from 1 forward and 1 reverse primer are termed 'cross-dimers'. Note that in reality primers would normally have around 20 bp, producing primer-dimers of up to 35 bp.

The reaction kinetics of a qPCR reaction are essentially the same as those of a classical PCR reaction, but amplification can usefully be classified into 4 phases (Figure 2.9, p 83) [Shipley, 2006]:

1. baseline phase: amplification is exponential, but is below the level of detection of the qPCR machine;

2. exponential phase: amplification is exponential and detectable above the background fluorescence;

3. linear phase: amplification is no longer exponential, but the amount of product continues to increase on a linear basis;

4. plateau phase: the rate of amplification starts to decline, and eventually amplification ceases completely.

Quantification in qPCR is achieved by comparing samples at a selected point in the exponential phase of the reaction, the quantification threshold, at which they all have the same amount of amplified DNA. The cycle (or, more accurately, the interpolated fractional cycle) at which the threshold is reached is termed the threshold cycle (C_T). The C_T value will vary between samples, and is linearly related to the logarithm of the initial number of template molecules in each sample [Bustin, 2012]. This relationship can be derived from Equation 2.2, p 75, which can be rearranged as:

$$N_0 = N_A/(1 + E)^n \tag{2.3}$$

where:

N_0 = initial number of molecules;
N_A = number of amplicons;
E = amplification efficiency;
n = number of amplification cycles.[23]
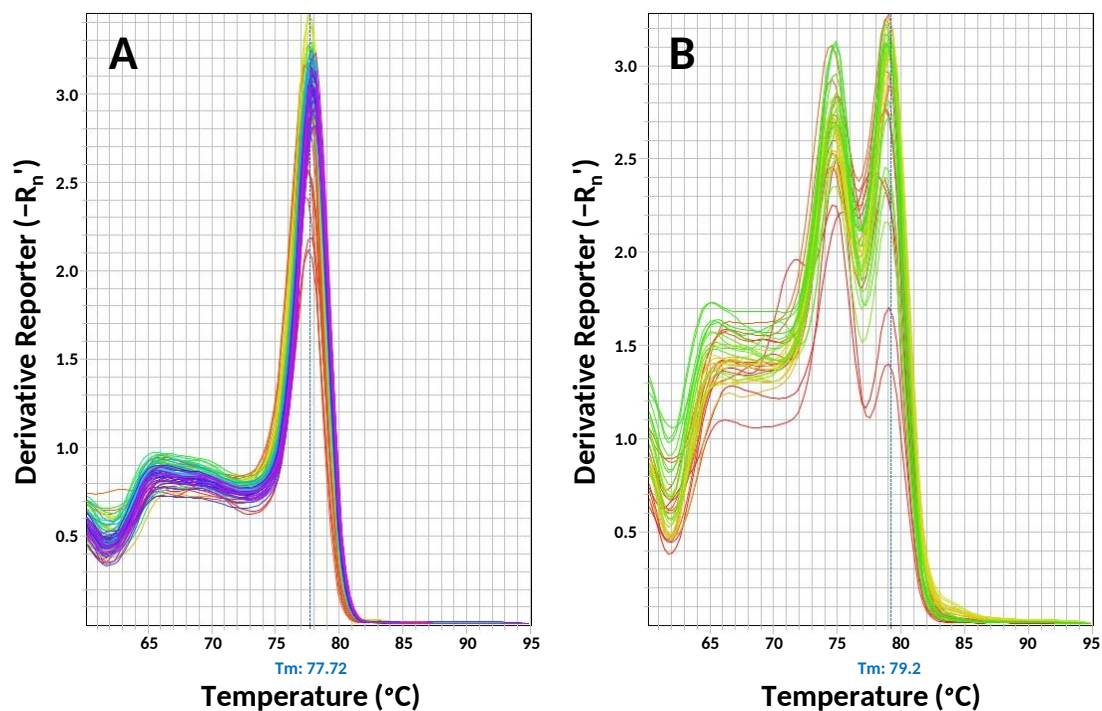
Figure 2.8 Examples of qPCR melt curves. The figures are images exported directly from the qPCR machine used in the study, an Applied Biosystems StepOnePlus. The curves show the melting temperature of the products in each of the utilised wells of the 96-well qPCR plate. The colours relate to the particular wells used, and have no other significance. **A.** This melt curve for insulin receptor (INSR) shows a clear single peak, indicating a single qPCR product; the primers used in this experiment are therefore fit for purpose. **B.** This melt curve for LHX8 varies from sample to sample and shows (at least) 2 peaks, indicating (at least) 2 qPCR products; the primers used in this experiment were subsequently rejected, and a new pair was designed. The 'U' shape and slight 'shoulder' on the left of the figures is a feature of the fast SYBR Green master mix used in the study.

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[23] The number of amplification cycles will be one less than the total number of cycles for single-stranded DNA.
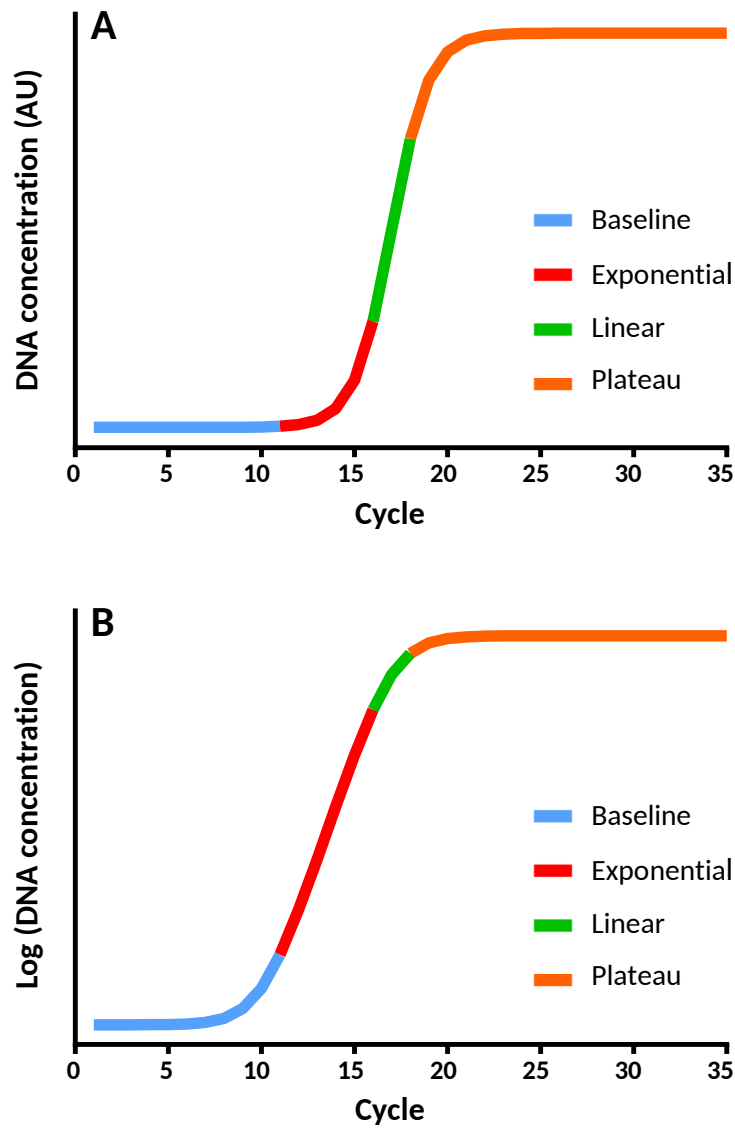
Figure 2.9 The 4 phases of amplification by qPCR. A. Linear. **B.** Logarithmic. 1. Baseline phase: amplification is exponential, but below the level of detection of the qPCR machine. 2. Exponential phase: amplification remains exponential, but is now detectable. 3. Linear phase: amplification is no longer exponential, but the amount of product continues to increase on a linear basis. 4. Plateau phase: the rate of amplification starts to decline rapidly, and eventually amplification ceases completely [Shipley, 2006]. The two graphs are very similar in shape, but the locations of the 4 phases on the two curves are different. For quantification of qPCR, measurements are taken at a threshold cycle (C_T) in the exponential phase. This can be difficult to judge on the linear graph, but is easily identifiable on the logarithmic graph as an ascending straight line.

As all samples are compared at a point where they have identical amounts of DNA, N_A is a constant, so Equation 2.3, p 82, can be restated as:

$$N_0 = N_T/(1 + E)^{C_T} \qquad\qquad (2.4)$$

where:

N_T = number of amplicons at the quantification threshold;

C_T = the threshold (or quantification) cycle.

Taking the logarithm of both sides of Equation 2.4, p 83, produces:

$$\log N_0 = -\log(1 + E)\, C_T + \log N_T \qquad (2.5)$$

which is in the form of the general equation for a straight line, $y = mx + c$ (where m is the slope and c is a constant corresponding to the intercept on the y-axis).

The linear relationship shown in Equation 2.5 can be used to determine the efficiency of a qPCR reaction, but first the equation needs to be rearranged to show C_T as a function of $\log N_0$:

$$C_T = \frac{-\log N_0}{\log(1+E)} + \frac{\log N_T}{\log(1+E)} \qquad (2.6)$$

The slope of the line produced by Equation 2.6 is obtained from the general equation for a straight line:

$$slope = \frac{-1}{\log(1+E)} \qquad (2.7)$$

The reaction efficiency can now be expressed in terms of the slope, assuming that all samples have the same efficiency, and that it is constant over the number of cycles needed to reach C_T:

$$E = 10^{-1/slope} - 1 \qquad (2.8)$$

A series of dilutions of a sample of template DNA of known concentration is prepared and then amplified by qPCR. Around 8 dilutions from a 10-fold series would be typical. The C_T value for each dilution is then plotted against the logarithm of the initial sample concentration (or initial cDNA copy number). A best-fit straight line, termed a 'standard curve', is then drawn through the plotted points, and the slope is measured (Figure 2.10, p 85). A perfect standard curve would have a slope of -3.32 (which correlates with 100 % efficiency), a y-axis intercept of 33–37 cycles, and a coefficient of determination (r^2) of 1. The y-axis intercept figures are derived from the fact that a single copy of a template DNA molecule should be detected at around 33.3–36.5 cycles [Scott Adams, 2006]. The r^2 value indicates how close a fit the straight line is to the plotted points. Typical values obtained in the study with dilutions from 10^8 down to 10^2 (and often 10^1) DNA copies were efficiencies of 90–100 %, r^2 values of 0.998–1, and y-intercept values of 35–39 cycles.

The first stage in qPCR quantification is to select the quantification threshold and then to determine the C_T values for each of the samples, standards and controls. The qPCR machine will produce an amplification plot for each sample of $\log \Delta R_n$ against cycle (Figure 2.11, p 86). The value ΔR_n is the normalised fluorescence corrected for background, and is therefore proportional to the amount of DNA. It is calculated as:

$$\Delta R_n = R_{n_{sample}} - R_{n_{background}} \qquad (2.9)$$
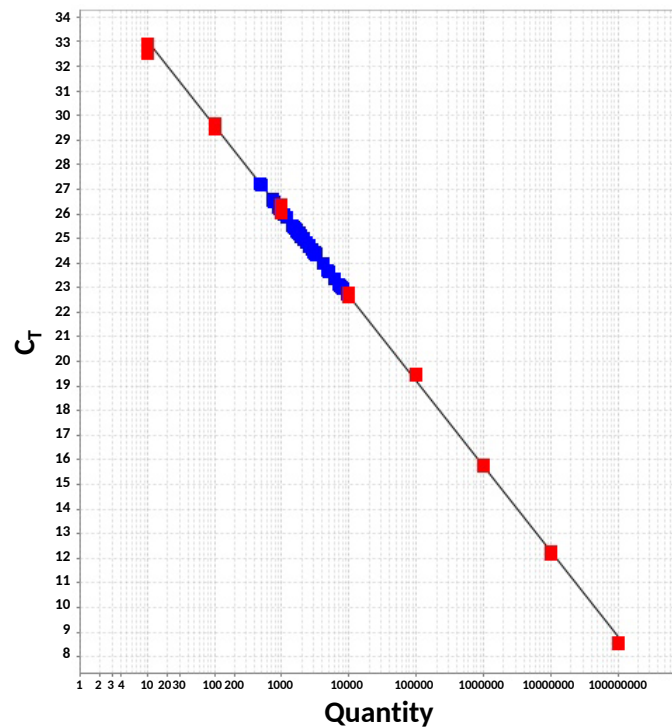
Figure 2.10 Example of a qPCR standard curve. The figure is an image exported directly from the qPCR machine used in the study, an Applied Biosystems StepOnePlus. The 'curve', which is in fact a straight line as the *x*-axis is logarithmic, shows the qPCR cycle (C_T) at which each sample from a set of 1 in 10 serial dilutions (red squares) reaches the threshold amplification. Three replicates were used for each dilution. In this case the quantity is expressed in terms of 'copy number', and the linear relationship is maintained to as few as 10 copies. (Below this figure replicates would be subject to sampling error.) The blue squares show where the samples tested would fall on the standard curve, thereby allowing their quantities to be determined by extrapolation. The gene in this case is insulin receptor (INSR).

where R_n is the normalised reporter signal, which is the fluorescence of the reporter dye (eg SYBR Green I) divided by the fluorescence of the reference dye (eg ROX) [Scott Adams, 2006]. The quantification threshold (shown by a horizontal green line in Figure 2.11, p 86) is automatically calculated by the qPCR machine, but it can be manually overridden. In practice, the actual threshold value selected is not particularly important, as long as it is in the exponential phase of amplification. If the reaction efficiency is the same for each sample, the amplification plots for each sample in the exponential phase of amplification will be parallel, and hence the difference in C_T values between samples will remain close to constant, irrespective of the quantification threshold chosen. The difference between C_T values of a dilution series should be constant (Figure 2.11, p 86), and corresponds to the slope of the standard curve (ie for a 10-fold dilution series with 100 % efficiency it will be approximately 3.32 cycles).

Although, from Equation 2.6, p 84, the C_T value of a sample is directly related to the logarithm of the initial number of template molecules, its value *per se* is meaningless; it is dependent on a number of factors, such as amplicon length, reaction efficiency and the
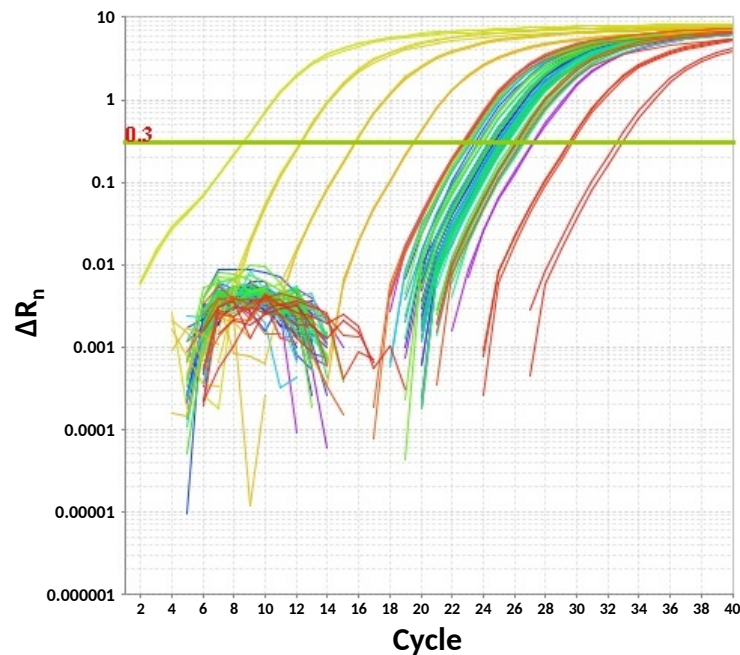
Figure 2.11 Example of a qPCR amplification plot. The figure is an image exported directly from the qPCR machine used in the study, an Applied Biosystems StepOnePlus. The y-axis is logarithmic, so the exponential phase of the amplification is an ascending straight line. The threshold (represented by a green horizontal line) is in the exponential phase. It has been chosen so that it is well clear of the baseline phase, but below the 'linear' phase where the curve starts to flatten (which would be linear in a non-logarithmic plot). As the curves are close to parallel in the exponential phase, the exact threshold value chosen is not important. The colours of the curves relate to the particular wells used on the qPCR plate; in this case, the yellow and red curves represent the serial dilutions that make up the standard curve (ideally these will be evenly spaced), and the green, blue and violet curves are samples. The gene in this case is insulin receptor (INSR).

quantification threshold selected. To be useful, therefore, it must be compared to something else: either the C_T values of a set of known quantities (absolute quantification); or the C_T value of another sample (relative quantification). Furthermore, variation in the reaction efficiency of RT leads to variation in the initial amount of cDNA in each sample,[24] and other factors can also affect the quantification of specific samples, including the integrity of the mRNA, the presence of PCR inhibitors, qPCR reaction efficiency, and small differences in temperature. It is therefore good practice to 'normalise' the measured quantity of DNA for a gene of interest in terms of the measured quantity of one or more reference (or 'house-keeping') genes that are stably expressed in the tissue of interest and that have been processed in the same way. Normalisation is desirable with both absolute and relative quantification.

For absolute quantification, serial dilutions of a known amount of cDNA are processed in the same qPCR run as the samples, and the resulting standard curve is used to inter-

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[24] It is not possible to measure the cDNA concentration after reverse transcription, because there is usually far more RNA than DNA present in the reaction tube, and there is no way to distinguish between the two with a spectrophotometer [Huggett *et al*, 2006].

polate a sample's initial cDNA concentration or copy number from its C_T value (Figure 2.10, p 85). However, the word 'absolute' must be treated with a degree of caution: firstly, the quantity figure obtained is always relative to something (eg the qPCR reaction volume, the concentration of mRNA, the mass of tissue, or the quantity of a reference gene); secondly, the efficiency of the RT reaction is not known, so the measured amount of cDNA for a gene cannot be directly related to the original amount of its mRNA; and finally, standards are prepared from purified cDNA, so the qPCR reaction efficiency of standards and samples (which could contain PCR inhibitors) is not necessarily the same. In spite of this proviso, absolute quantification does provide a means to compare the relative levels of expression in a tissue of different genes of interest, and to assess whether relative differences in the expression of a particular gene are likely to be physiologically or metabolically relevant (eg a two-fold change from 100 000 to 200 000 DNA copies is likely to be more important than a two-fold change from 10 to 20 copies). Another advantage is that it provides a means to quantify any contamination in negative controls. Furthermore, use of absolute quantification does not preclude the use of relative quantification.

For relative quantification, one sample or, more usually, one experimental group is chosen as a 'calibrator', and the amount of cDNA of other samples or groups is expressed relative to that of the calibrator. Sometimes the choice of calibrator is obvious (eg the control condition is likely to be chosen as the calibrator in a drug trial), in other cases it is purely subjective. The relative quantity is in effect the ratio of two ratios: the normalised value of the sample to the normalised value of the calibrator. There are 3 main methods of relative quantification:

- the relative standard curve method [Larionov *et al*, 2005];

- the Pfaffl method [Pfaffl, 2001];

- the $2^{-\Delta\Delta C_T}$ method [Livak & Schmittgen, 2001].

The relative standard curve method is essentially the same as absolute quantification, except that the amounts of cDNA are presented relative to those of a calibrator instead of in terms of normalised copy number. Its main advantage is that there is no requirement to calculate (or assume) a reaction efficiency.

The Pfaffl method uses reaction efficiencies to calculate the relative quantity, and the equation used for this method can be derived from Equation 2.4, p 83 [Pfaffl, 2001]:

$$R = \frac{(1+E_{GOI})^{\Delta C_{T\,GOI}}}{(1+E_{Ref})^{\Delta C_{T\,Ref}}} \tag{2.10}$$

where:

R = relative quantity;

E_{GOI} = reaction efficiency of the gene of interest (GOI);

E_{Ref} = reaction efficiency of the reference gene;

$\Delta C_{T_{GOI}}$ = difference in C_T value between calibrator and sample for the GOI;

$\Delta C_{T_{Ref}}$ = difference in C_T value between calibrator and sample for the reference gene.

With multiple samples in an experimental group, the mean C_T value for each sample and calibrator group are used in calculating $\Delta C_{T_{GOI}}$ and $\Delta C_{T_{Ref}}$. As with absolute quantification and the relative standard curve method, serial dilutions of a known amount of cDNA are processed in the same qPCR run as the samples, and Equation 2.8, p 84, is used to calculate the reaction efficiency from the slope of the resulting standard curve.

The $2^{-\Delta\Delta C_T}$ method is an idealised version of the Pfaffl method in which the efficiencies of both the gene of interest and reference gene(s) are assumed to be 100 %. Substituting 1 for E in both the numerator and denominator, Equation 2.10, p 87, becomes:

$$R = 2^{-\left(\Delta C_{T_{sample}} - \Delta C_{T_{calibrator}}\right)} = 2^{-\Delta\Delta C_T} \qquad (2.11)$$

where:

$\Delta C_{T_{sample}}$ = difference in sample C_T values between GOI and reference gene;

$\Delta C_{T_{calibrator}}$ = difference in calibrator C_T values between GOI and reference gene.

For the $2^{-\Delta\Delta C_T}$ method to be valid, the reaction efficiencies of the gene of interest and reference gene(s) must be approximately equal [Livak & Schmittgen, 2001]. This is because small differences in efficiency can produce large differences in quantity at the threshold cycle (Table 2.2, p 89). Its main advantage is that there is no requirement to run serial dilutions on every run, thereby freeing up more wells of a 96-well microtitre plate for samples. However, if no standard curve is produced, there is no way of ascertaining whether the primers and/or the cDNA have deteriorated.

Absolute quantification and all 3 methods of relative quantification can be adjusted for normalisation with multiple reference genes [Vandesompele *et al*, 2002]. In this case, a reference index is produced from the geometric mean expression of the selected reference genes. The geometric mean is used rather than the arithmetic mean because it is not biased by differences in levels of absolute expression between the genes.

2.4.3 Tissue homogenisation and RNA extraction

The amount of tissue used and method of RNA extraction was different for the two studies. With Study A, there was plenty of tissue available, so the principal consideration was to agree a method of RNA extraction with colleagues who were working on different tissues

Table 2.2 Effect of reaction efficiency on DNA copy number. Each column shows the DNA copy number at the end of each cycle for a different level of reaction efficiency. The bottom line shows the relative amounts after 20 cycles.

Cycle	Copy number					
	100 %	95 %	90 %	85 %	80 %	75 %
0	1	1	1	1	1	1
1	2	2	2	2	2	2
2	4	4	4	3	3	3
3	8	7	7	6	6	5
4	16	14	13	12	10	9
5	32	28	25	22	19	16
6	64	55	47	40	34	29
7	128	107	89	74	61	50
8	256	209	170	137	110	88
9	512	408	323	254	198	154
10	1 024	795	613	470	357	269
11	2 048	1 550	1 165	869	643	471
12	4 096	3 023	2 213	1 607	1 157	825
13	8 192	5 895	4 205	2 973	2 082	1 444
14	16 384	11 494	7 990	5 501	3 748	2 527
15	32 768	22 414	15 181	10 176	6 747	4 422
16	65 536	43 707	28 844	18 826	12 144	7 738
17	131 072	85 229	54 804	34 827	21 859	13 541
18	262 144	166 197	104 127	64 430	39 346	23 697
19	524 288	324 084	197 842	119 196	70 824	41 469
20	1 048 576	631 964	375 900	220 513	127 482	72 571
Rel	1.00	0.60	0.36	0.21	0.12	0.07

from the same animals. With Study B, only a limited amount of tissue was received from Denmark, so the principal consideration was to find a method of RNA extraction that was suitable for relatively small amounts of adipose tissue. RNeasy kits (QIAGEN, Crawley, West Sussex, UK) were used for RNA extraction in both studies, but the specific kit used was different for the two studies, and there was some modification to the manufacturer's protocol at the tissue homogenisation stages. Furthermore, for Study A, additional steps (developed in our laboratory over time) were included after the homogenisation stage to maximise the removal of lipid from the homogenate.

2.4.3.1 Study A

RNA was extracted using an RNeasy Plus Mini Kit (QIAGEN). Approximately 250 mg tissue was chopped, weighed, placed in a tissue dissociation tube (gentleMACS; Miltenyi Biotec Ltd, Bisley, Surrey, UK), and homogenised in 1 ml TRI-Reagent using a Dispomix Drive

(Medic Tools, Zug, Switzerland). The tubes were then centrifuged at 1 800 xg for 30 s at 4 °C, floated in a water bath at 37 °C for 2 min, vortexed 5 times over 30 s, and centrifuged at 1 800 xg for 5 min at 4 °C. The lower (aqueous) phase of the homogenate was drawn off into a 2 ml Eppendorf tube, and 200 µl chloroform was added to it. The resulting mixture was vortexed, incubated for 10 min at room temperature, and centrifuged at 18 000 xg for 15 min at 4 °C. The remainder of the protocol was in accordance with the manufacturer's instructions.

2.4.3.2 Study B

RNA was extracted using an RNeasy Universal Mini Kit (QIAGEN). Some 100 mg tissue was chopped, weighed, placed in a gentleMACS tissue dissociation tube, and homogenised in 900 µl QIAzol Lysis Reagent (QIAGEN) using a Dispomix Drive. The tubes were centrifuged at 1 800 xg for 30 s at 4 °C, and the homogenate drawn off into 2 ml Eppendorf tubes. The remainder of the protocol was in accordance with the manufacturer's instructions.

2.4.4 Quantification of RNA and assessment of its purity

The concentration of RNA extracted from each tissue sample was measured, so that the same amount of RNA from each sample could be used for RT. Measurements were made on a NanoDrop ND-1000 spectrophotometer (Thermo NanoDrop, Wilmington, USA). If initial concentrations were too high, the samples were diluted with nuclease-free water (QIAGEN) and the measurements repeated, until the final RNA concentrations were reduced to below 1 000 ng µl^{-1}.

The maximum absorption of nucleic acids is at 260 nm, and the intensity of absorption at this wavelength is used to determine their concentration. The instrument also displays two absorbance ratios which are used as an indication of the purity of a sample: 260 nm to 280 nm (the 260:280 ratio) and 260 nm to 230 nm (the 260:230 ratio). The 260:280 ratio should be around 2.0 for RNA and 1.8 for DNA. Figures lower than these may indicate the presence of phenol (from QIAzol or TRI-Reagent) and/or other contaminants that absorb at or close to 280 nm, though the figures are dependent on the solution pH and the exact mix of nucleotides. The 260:230 ratio should be in the region 2.0–2.2, but there is no consensus on an acceptable lower limit, and the value is affected by the RNA concentration. Figures lower than the quoted range may indicate the presence of phenolate ions, guanidine isothiocyanate (GITC; present in lysis buffers or extraction reagents), carbohydrates and/or other contaminants that absorb at or close to 230 nm. GITC is a major cause of absorption at 230 nm, but does not compromise the reliability of RT-PCR.

For Study B sternal adipose tissue, RNA extractions were not repeated for samples with low 260:280 and/or 260:230 ratios because of a lack of appreciation at the time of the relevance of these ratios. For Study B subcutaneous adipose tissue and Study A sternal adipose tissue, up to two further extractions of RNA were made from samples with poor 260:280 and/or 260:230 ratios. If acceptable ratios had not been achieved after three attempts, then the 'purest' extraction was selected for each sample. As can be seen from Table 2.3, below, the 260:280 ratios were acceptable for all the study tissues. The 260:230 ratios were less satisfactory, being particularly poor in some of the samples from Study B sternal adipose tissue. The re-extractions improved the situation with the other tissues, with the 260:230 ratios for Study A sternal adipose tissue being close to ideal in most cases. It should be noted that poor 260:230 ratios will not necessarily be reflected in PCR performance, and there was no subsequent indication that amplification of samples with poor 260:230 ratios was compromised. Finally, it is emphasised that both RNA and DNA absorb maximally at 260 nm, so RNA and DNA cannot be distinguished by a spectrophotometer.

Table 2.3 Spectrophotometer RNA purity ratios. Number of samples and average, median, maximum and minimum 260:280 ratios and 260:230 ratios and standard deviation (SD) for RNA extracted from tissues for Studies A and B. These ratios give an indication of the purity of the RNA. The 260:280 ratio should be around 2.0 for RNA, though the figures are dependent on the pH of the solution and the exact mix of nucleotides. Values less than these figures may indicate the presence of phenol (from extraction reagents) and/or other contaminants that absorb at or close to 280 nm. The 260:230 ratio is a secondary measure of RNA purity and should be in the range 2.0–2.2, though there is no consensus on an acceptable minimum, and the ratio is dependent on RNA concentration. Values less than 2.0 may indicate the presence of phenolate ions, guanidine isothiocyanate (GITC; from lysis buffers or extraction reagents), carbohydrates and/or other contaminants that absorb at or close to 230 nm. GITC is a major cause of absorption at 230 nm, but does not compromise the reliability of RT-PCR. 'Sternal' is sternal adipose tissue; 'Subcut' is subcutaneous adipose tissue.

| Value | 260:280 ratio | | | 260:230 ratio | | |
| | Study A | Study B | | Study A | Study B | |
	Sternal	Sternal	Subcut	Sternal	Sternal	Subcut
Number	54	26	25	54	26	25
Mean	2.12	2.04	2.04	2.11	1.68	1.74
SD	0.05	0.06	0.04	0.13	0.42	0.21
Median	2.13	2.03	2.05	2.14	1.83	1.77
Maximum	2.19	2.14	2.09	2.27	2.10	2.00
Minimum	1.93	1.92	1.95	1.64	0.54	1.27

2.4.5 Analysis of RNA integrity

No attempt was made initially to analyse the integrity of the RNA extracted from Study B sternal adipose tissue samples, but for the other tissues (processed later) it was felt that this would improve the overall protocol. There are various instruments and methods used to analyse RNA integrity, but in general they look for the presence of two prominent peaks or bands corresponding to 18S and 28S ribosomal RNA (rRNA), as some 95 % of cellular RNA is ribosomal. The method used in the study was to subject a number of samples of RNA to electrophoresis on a non-denaturing agarose gel. Around 3 µg RNA in 15 µl nuclease-free water (QIAGEN) was used for each sample, with 5 µl loading dye (Certistain, Orange G; Merck, Darmstadt, Germany) being added to make up a total volume of 20 µl. The samples were run on a 1 % gel, being 0.5 g agarose (Fisher Scientific, Loughborough, UK) in 50 ml tris-acetate-ethylenediaminetetraacetate (TAE) buffer, with 2.5 µl ethidium bromide added to the gel mix. A 100 bp DNA ladder (GeneRuler; Fisher Scientific) was used as a marker, as there were no RNA ladders in stock. In other respects, the procedure was the same as that used for DNA agarose gel electrophoresis (Section 2.4.9.2, p 96).

RNA was extracted from Study B subcutaneous fat samples in 5 separate extractions (including repeats) over a period of a few days, so one sample was selected from each of the 5 extractions. The results were reasonable, with two clear bands at about 1 700 and 800 DNA bp corresponding to 28S and 18S rRNA respectively (Figure 2.12, below).[25] Usually the 28S band would be expected to be about twice the intensity of the 18S band (which it was not), but this can vary according to the species, tissue type and extraction method.

The first agarose gel run with RNA extracted from Study A sternal adipose tissue was
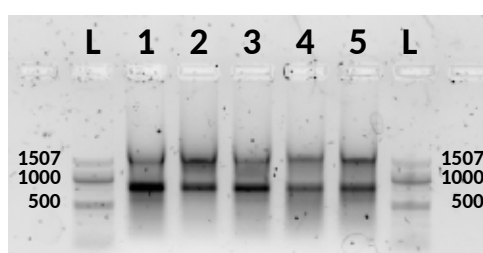
Figure 2.12 Agarose gel of RNA extracted from Study B subcutaneous adipose tissue. Test of integrity of RNA extracted from 5 samples. Bands at around 1 700 and 800 DNA base pairs correspond to 28S and 18S ribosomal RNA (rRNA) respectively. Prominent bands of this size with minimal smearing indicate good quality RNA. Usually the 28S band would be expected to be about twice the intensity of the 18S band, but this can vary according to the species, tissue type and extraction method. L is DNA ladder (used because there were no RNA ladders in stock); numbers are samples.

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[25] The 28S and 18S ribosomal bands would be expected to be around 4 000–5 000 bp and 1 800–2 000 bp respectively on an RNA ladder.
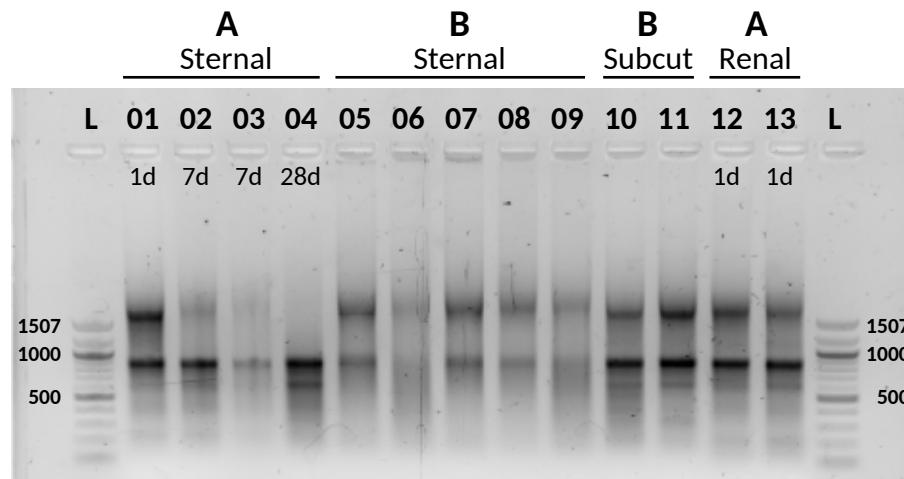
Figure 2.13 Agarose gel of RNA extracted from samples of Studies A and B sternal adipose tissue. Test of integrity of RNA extracted from 4 samples of Study A sternal adipose tissue and 5 samples of Study B sternal adipose tissue. RNA extracted from 2 samples of Study B subcutaneous adipose tissue and 2 samples of perirenal adipose tissue from the Study A animals were used as positive controls. Bands at around 1 700 and 800 DNA base pairs (bp) correspond to 28S and 18S ribosomal RNA (rRNA) respectively. Prominent bands of this size with minimal smearing indicate good quality RNA. Usually the 28S band would be expected to be about twice the intensity of the 18S band, but this can vary according to the species, tissue type and extraction method. L is DNA ladder (used because there were no RNA ladders in stock). The 2-digit numbers at the top of each column are sample numbers. 1d, 7d and 28d are 1 day-old, 7 day-old and 28 day-old tissues respectively. A is Study A; B is Study B. 'Sternal' is sternal adipose tissue; 'Subcut' is subcutaneous adipose tissue; 'Renal' is perirenal adipose tissue. There was slight spillage when pipetting sample 03 into the gel, which probably explains the less intense 18S band. The absence of a 28S ribosomal band in the Study A sternal adipose tissue 7 day-old and 28 day-old samples is consistent with another sheep study being undertaken in our laboratory.

unsatisfactory, with virtually no clear bands, and smearing in all samples (not shown). A second gel was therefore run with 4 samples from Study A sternal adipose tissue, 5 samples from Study B sternal adipose tissue (not previously assessed), and 4 positive controls: 2 from Study B subcutaneous adipose tissue and 2 from the perirenal adipose tissue of Study A animals (Figure 2.13, above). The positive controls all had 2 clear bands at around 1 700 and 800 DNA bp, corresponding to the 28S and 18S ribosomal bands, though the latter band was more intense in all cases. There were two clear bands in 4 of the 5 Study B (6 month-old) sternal adipose tissue samples, but these were much less clearly defined than in the positive controls. The 4 Study A sternal adipose tissue samples all had a clear band at around 800 DNA bp corresponding to the 18S ribosomal band. The 1 day-old sample also had a clear intense band at around 1 700 DNA bp, corresponding to the 28S ribosomal band, but this had all but disappeared in the 7 and 28 day-old samples. A colleague subsequently tested samples of perirenal and sternal adipose tissue from an unrelated sheep study using a similar protocol to Study A, and found that the RNA extracted from perirenal adipose tissue had two clear bands, but that RNA extracted from sternal adipose tissue, as with

Study A, was missing the 28S band. It may be, therefore, that the 'disappearing' 28S band in sternal adipose tissue is a function of the extraction method. In any event, degradation of rRNA is not necessarily an indication of degradation of the much shorter mRNA and, as has been previously noted, results can vary by tissue type. It was therefore decided to proceed with the existing RNA for Study A sternal adipose tissue. There was no subsequent indication that amplification of Study A sternal adipose tissue samples had been compromised.

2.4.6 Additional DNase treatment of RNA

Both the RNeasy Universal Mini Kit and RNeasy Plus Mini Kit used for RNA extractions include constituents to eliminate gDNA. However, in the pilot stage of the study there was apparent contamination of samples by gDNA.[26] It was therefore decided to further treat the extracted RNA with a DNase to remove any residual gDNA. The kit used was RQ1 RNase-Free DNase (Promega, Southampton, UK). The appropriate volume of RNA solution required to produce 1 µg (Study A) or 0.9 µg (Study B) RNA was calculated for each sample and transferred to a 200 µl Eppendorf tube, and nuclease-free water was then added to bring the total volume up to 8 µl. This was followed by the addition of 1 µl RQ1 DNase and 1 µl RQ1 10x Reaction Buffer to make up a total reaction volume of 10 µl, which was then processed in accordance with the manufacturer's instructions. The reaction was ultimately terminated by the addition of 1 µl Stop Solution, to give a final volume of 11 µl.

2.4.7 cDNA preparation

RT was performed using an Applied Biosystems High Capacity RNA-to-cDNA Kit (Life Technologies, Paisley, UK). The manufacturer's protocol requires 9 µl of RNA solution to make up a total reaction volume of 20 µl. In order to accommodate the 11 µl RNA solution obtained after DNase treatment (Section 2.4.6, above), a slight adjustment was made to the protocol such that the active reagents were pro-rated by a factor of 5/4 to make up a total reaction volume of 25 µl, with the difference being made up by nuclease-free water. The final reaction mix in each 200 µl Eppendorf tube was therefore 11 µl RNA solution, 12.5 µl 2x RT Buffer, 0.25 µl nuclease-free water and 1.25 µl 20x RT Enzyme Mix. The reaction mixture was processed in accordance with the manufacturer's instructions, with thermal cycling being carried out on a TouchGene Gradient (Techne, Duxford, Cambridgeshire, UK). After completion of RT, samples were diluted by 1 in 8 to produce a 200 µl stock of cDNA solution for each sample. For Study B sternal adipose tissue, the dilution was in nuclease-

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[26] This is now actually believed to be the presence of primer-dimers in samples with low amounts of template DNA.

free water. For Study B subcutaneous adipose tissue and Study A sternal adipose tissue, the dilution was in a 10 ng µl^{-1} solution of yeast transfer RNA (tRNA). The addition of tRNA as a 'carrier' stabilises low copy number cDNA. In addition to the samples, a number of non-reverse transcriptase controls (NRTCs) and an NTC were also processed. An NRTC is a sample which contains RNA plus all the components of the reaction mixture except the RT enzyme. An NTC contains all the reaction mixture, including the RT enzyme, but excludes any RNA. In both cases the difference in volume was made up by nuclease-free water. If not required immediately, cDNA solutions were stored at –20 °C.

2.4.8 Extraction of gDNA

Genomic DNA was used as a control for testing primers, specifically to ascertain whether potential primers would amplify genomic products of a similar size to the target amplicon. It was extracted from the 1 day-old muscle and perirenal adipose tissue of sheep used in a previous study in our laboratory, using a DNeasy Blood & Tissue Kit (QIAGEN). A number of samples from each tissue were cut and processed. For each sample, around 25 mg tissue was chopped, weighed, placed in a gentleMACS tissue dissociation tube and homogenised in 180 µl Buffer ATL using a Dispomix Drive. The tubes were centrifuged at 1 800 xg for 30 s at room temperature. The homogenate was drawn off into 1.5 ml Eppendorf tubes, to which 20 µl Proteinase K was added. The remainder of the protocol was in accordance with the manufacturer's instructions. At the end of the process, the samples were combined and mixed, the DNA concentration was measured on a NanoDrop ND-1000 spectrophotometer, and 200 µl aliquots of 100 ng µl^{-1} gDNA were prepared by dilution in nuclease-free water.

2.4.9 Classical PCR and cDNA extraction from gels

Classical PCR was used (1) to test potential primers and (2) to produce concentrated cDNA from which serial dilutions could be prepared for standard curves. The PCR products were subjected to agarose gel electrophoresis, imaged, and, if the primers were considered to be acceptable, the appropriate 'band' was cut out from the gel and the cDNA was extracted.

2.4.9.1 Classical PCR

A reaction volume of 20 µl, comprising 10 µl DNA polymerase (One Taq Hot Start Quick-Load 2X Master Mix with Standard Buffer; NEB, Hitchin, Hertfordshire, UK), 4 µl each of 2.5 µM forward and reverse primers, and 2 µl cDNA solution, was dispensed into 200 µl Eppendorf tubes and mixed by pipetting. The tubes were briefly spun in a microcentrifuge, then transferred to the Touchgene Gradient thermal cycler. Samples were heated to 94 °C

for 30 s to activate the DNA polymerase, then submitted to 40 cycles of melting, annealing and extension at 94 °C for 30 s, 60 °C for 30 s and 68 °C for 1 min respectively. There was a final extension period of 5 min at 68 °C, after which the samples were held at 8 °C.

2.4.9.2 Agarose gel electrophoresis and gel cutting

Amplified PCR products were run on 3 % agarose gels using a GeneRuler Low Range DNA ladder (Fisher Scientific). Gels were prepared by adding 1.5 g of agarose powder (Fisher Scientific) to 50 ml of 1X TAE buffer, heating the mixture until the powder was dissolved, cooling the solution, adding 2.5 µl of 10 mg ml^{-1} ethidium bromide, and then pouring the solution into a cassette to set. Once the gel was set, the cassette was placed in a tank filled with 1X TAE buffer, 5 µl of DNA ladder was loaded into the first well, the full amount of each PCR product (20 µl) was loaded into the other wells, and electrophoresis was initiated at 80 V. The DNA polymerase mix contains a tracking dye, so there was no requirement for a separate loading dye. Gels were imaged in a Fujifilm LAS-3000 Luminescent Imager Analyzer (Raytek, Sheffield, UK). Bands corresponding to the appropriate amplicon length were then cut out of the gel and stored in 1.5 ml Eppendorf tubes at –20 °C pending cDNA extraction.

2.4.9.3 cDNA extraction from excised gel

The cDNA was extracted from the excised bands of gel using the QIAquick Gel Extraction Kit (QIAGEN) in accordance with the manufacturer's instructions. The cDNA concentration was measured with a NanoDrop ND-1000 spectrophotometer, and the cDNA was stored in 1.5 ml Eppendorf tubes at –20 °C.

2.4.10 Primer design and testing

2.4.10.1 Introduction

Primer sequences for genes used in the study were obtained from a number of different sources: our database of well-established primers; the literature; and a colleague, Mr Mark Pope, who had recently designed primers for some of the genes of interest. Where no sequences were available, or existing primers were deemed unsatisfactory, new primers were designed.

2.4.10.2 Primer design

For the first two years of the study, there was no published sheep (*Ovis aries*) genome, so primers were designed to the cow (*Bos taurus*). The first draft of the sheep genome became

available in the main online databases in around October 2013, in advance of the paper that formally announced the sequencing, which was published the following June [Jiang *et al*, 2014]. From the end of 2013 onwards, primers were designed to the sheep genome.

The parameters for good primer design vary slightly from source to source, but a reasonable consensus is as follows [Bustin & Nolan, 2006; McPherson & Møller, 2006; Wang & Seed, 2006]:

- primers should be 16–28 nucleotides long;

- amplicons should be 70–250 bp long;

- the melting temperatures (T_m) for both primers should be in the range 55–60 °C; and should not differ by more than 2 °C;

- the GC (guanine/cytosine) content should be 40–60 %;

- long runs (ie more than 3) of a single base, especially G or C, should be avoided;

- repeated sequences should be avoided;

- complementary sequences should be avoided, especially at the 3'-ends;

- secondary structure in either the primers or the amplicon should be avoided;

- primers should be either intron flanking or exon spanning.

The last requirement is intended to detect or prevent the amplification of residual gDNA. A gene in its genomic form is (usually) made up of a number of coding exons interspersed with a number of non-coding introns: in its transcribed form it consists of a sequence of spliced exons. This difference between cDNA and gDNA gives rise to 3 different types of primer pairs: single exon; intron flanking; and exon spanning (Figure 2.14, p 98). With single exon primers, the forward and reverse primers bind to the same exon. In this case, the product from both cDNA and any residual gDNA is the same, so there is no way to detect contamination of samples with gDNA. With intron flanking primers, the forward and reverse primers bind to different exons. The genomic product will therefore have at least one intron between the primer binding sites, so any residual gDNA product will be longer than the cDNA amplicon. This may prevent amplification completely (ie if the intron is longer than about 1 000 bp), or allow the presence of any genomic products to be detected on a gel or by qPCR melt curve analysis. With exon spanning primers, one of the primers is designed so that it spans the boundary between two exons. The equivalent sequence in gDNA will be interrupted by an intron, so the exon-spanning primer will not bind to it. There should therefore be no genomic products.
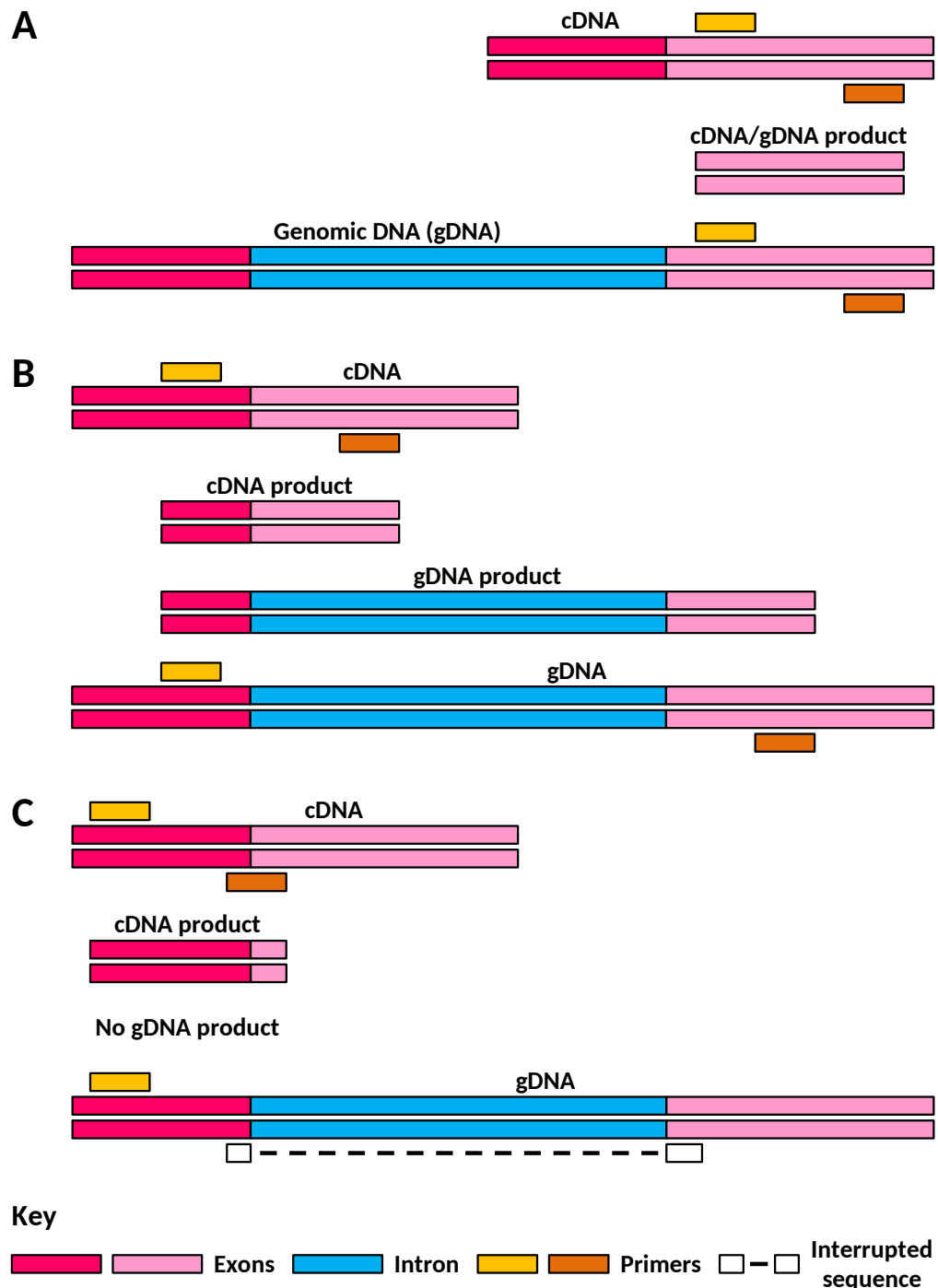
Figure 2.14 The 3 types of PCR primer pairs. A simple gene with 2 exons and 1 intron is used as an example. **A.** Single exon primers: both primers fall within a single exon. The product from both complementary DNA (cDNA) and any residual genomic DNA (gDNA) is the same, so there is no way to detect contamination of samples with gDNA. **B.** Intron flanking primers: primers are designed so that the forward and reverse primers bind to separate exons. Any genomic product would therefore have at least one intron between the primer binding sites, so it would be longer than the cDNA amplicon. This may prevent gDNA amplification completely (ie if the intron is longer than around 1 000 bp), or allow the presence of any genomic products to be detected on a gel or by qPCR melt curve analysis. **C.** Exon spanning primers: one of the primers is designed so that it spans the boundary between 2 exons. The complementary sequence in gDNA will be interrupted by an intron, so the primer will not bind to it. There should therefore be no genomic products. Ideally primers are designed to be either intron flanking or exon spanning.

Primers were designed using an online tool, NCBI Primer-BLAST [NCBI, 2014c; Ye *et al*, 2012]. Default settings were used mainly, though the amplicon size was set at 70–150 bp initially, with the upper limit being increased to 200 and 250 bp if necessary. The software produces a list of potential primers and 'blasts' them against the appropriate genome to ascertain whether there are any predicted unintended targets. Primers were only selected if they had very few (preferably zero) unintended targets, or if the products of unintended targets was of a size unlikely to be amplified by PCR. The software also has the facility to select intron flanking or exon spanning primers, so in general the strategy was to design two pairs of potential primers at a time (primers being relatively cheap), one being intron flanking and the other exon spanning, with a view to testing and selecting the best pair. This was not always possible: sometimes the sequences of genes of interest were such that the software could not find good intron-flanking and/or exon-spanning primers (ie the parameters for good design would have been compromised), in which case single exon primers might have to be accepted. Furthermore, one of the genes of interest examined in the study, RIP140, only has a single exon. A further strategy was to try to design primers such that their amplicon matched sequences published by both the main online databases, NCBI [NCBI, 2014b] and Ensembl [Ensembl, 2014]. Again, this was not always possible, and even when it was, there were occasionally differences in the sequence and/or lengths of amplicons predicted by the two databases. In the later stages of the study, two more online sites were used to test potential primers for predicted secondary structure: PCR Primer Stats [Bioinformatics, 2014] and the DINAMelt web server [RNA Institute, 2014].

2.4.10.3 Purchase and preparation of primers

Primer pairs were ordered in dry powder form and made up to a 100 M stock solution with nuclease-free water. Stock solutions were diluted 1:40 into 2.5 M aliquots for use in PCR.

2.4.10.4 Primer testing

Potential primers, whether designed or obtained from other sources, were tested by using them for classical PCR with suitable sample tissues, negative controls and gDNA. They were rejected unless there was a single clear band on the gel corresponding to the expected amplicon size, negative control lanes were clear (except possibly for primer-dimers), and any products from amplification of gDNA could be easily distinguished from the target (ie they were not close to the amplicon size).

In spite of the strategy explained above, the design of primers was very much a 'hit and miss' process, with a success rate probably in the order of 1 in 5. Designing to the cow genome (or an early draft of the sheep genome) was obviously a factor. Failures included

no products at all, amplification of multiple products, primer-dimers in the sample lanes of gels, and unexpected amplification in negative control and/or gDNA lanes (Figure 2.15, below). The last category included amplicon-sized products where none should have been produced (ie the primers were designed to be intron-flanking or exon-spanning). Possible reasons for this are that the exon/intron split for the gene in the published databases was inaccurate, that negative controls and/or gDNA had been contaminated with cDNA from a sample, or that the genome contained a 'processed pseudogene' for the gene of interest [Wang & Seed, 2006]. Processed pseudogenes are non-functional sequences of mRNA which have been reincorporated in whole or part into the genome [Vanin, 1985]. They are much more common than perhaps is generally realised; for example, the human genome has around 8 000 [Zhang et al, 2003]. Whatever the cause, the best solution is to minimise the presence of any residual gDNA, so the double DNase treatment explained in Section 2.4.6, p 94, was probably fortuitous. The sequences and sources of primers for all the genes used in the study are shown in Table 2.4, p 101.
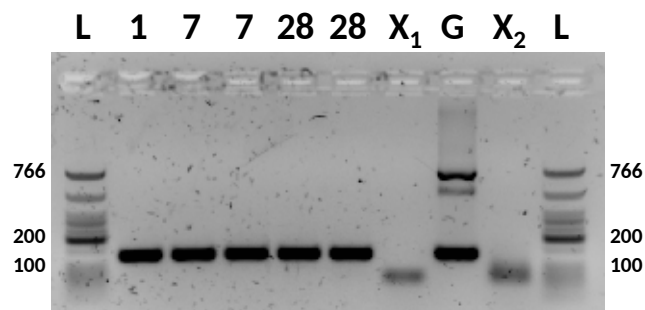
Figure 2.15 Test of potential primers showing the presence of an unexpected product in the gDNA lane. The sequences for this gene, RPL19, were obtained from a 2011 paper on evaluation of reference genes for ovine blood [Peletto et al, 2011]. The predicted amplicon size was 126 base pairs (bp). The primers were designed to flank an intron of 622 bp, giving an expected genomic product of 748 bp. The five sample lanes (from Study B tissues) show a clear single band of about the predicted amplicon size. The two negative control lanes have no band of the predicted amplicon size, but do have fainter bands of a smaller size which are probably primer-dimers. The gDNA lane shows a band of about the size of the predicted genomic product, just below the 766 bp marker, and another fainter band at around 600 bp. However, by far the most prominent band is of about the size of the predicted amplicon. As the primers are intron flanking, there should be no amplicon-sized product in the gDNA lane. A possible explanation is that the genome contains a processed pseudogene for RPL19. In the event, these primers were not used, as any contamination of samples by gDNA would not have been detected. L is DNA ladder; 1 is a 1 day-old sample; 7 is a 7 day-old sample; 28 is a 28 day-old sample; X_1 is the NRTC; G is gDNA; X_2 is the NTC. RPL19, a potential reference gene, was ultimately not used in the study.

Table 2.4 Primer sequences. Sources: previously published (article); designed by the author (MDAB) using the NCBI Primer-BLAST tool [NCBI, 2014c; Ye *et al*, 2012]; designed by a colleague, Mark Pope (MP), using the Primer3 tool [Koressaar & Remm, 2007; Primer3 Development Team, 2014; Untergasser *et al*, 2012]. Types: both primers bind to a single exon (E); one of the primers spans an exon-exon boundary (EE); the forward and reverse primers bind to separate exons which flank one or more introns (EIE); not known (NK). Length is the amplicon length in base pairs. The full name of each of the genes listed is shown in the List of Abbreviations and Acronyms (p 15).

Gene	Source	Type	NCBI reference	Ensembl reference	Forward primer sequence	Reverse primer sequence	Length
18S	article[a]	NK	NR_046261.1	NA	GATGCGGCGGCGTTATTCC	CTCCTGGTGGTGCCCTTCC	126
ADIPOQ	article[b]	E	NM_174742.2	ENSBTAT00000026395	ATCAAACTCTGGAACCTCCTATCTAC	TTGCATTGCAGGCTCAAG	232
ATF2	MDAB[c]	EE[h]	XM_004004570.1	ENSOART00000000205	TCCCACTTGTTCGACCAGTCA	TTGACAGTATCGCCGTTGGT	151
C/EBPα	MP[c]	E	XM_004015623.1	ENSOART00000004803	CTGGAGCTGACCAGTGACA	GGGCAGCTGACGGAAGAT	96
CIDEA	MDAB[d]	EIE	NM_001083449.1	ENSBTAT00000064010	AAGGCCACCATGTACGAGAT	GGTGCCCATGTGGATAAGACA	138
DIO2	article[b]	EIE	NM_001010992.3	ENSBTAT00000002107	AGCCGCTCCAAGTCCACTC	TTCCACTGGTGTCACCTCCT	175
FABP4	article[b]	EIE	NM_174314.2	ENSBTAT00000000079	TGAAATCACTCCAGATGACAGG	TGGTGGTTGATTTTCCATCC	98
GPR120	MDAB[d]	EIE	XM_002698388.1	ENSBTAT00000000560	CCTGGGACGTGTCATTTGCTA	CTGGTGGCTCTCCGAGTAGG	140
HOXC9	article[b]	EE	XM_002704244.2	ENSBTAT00000007361	GACCTGGACCCCAGCAAC	GCTCGGTGAGGTTGAGAAC	175
INSR	article[e]	EIE	XM_002688832.3	ENSBTAT00000016858	CTGCACCATCATCAACGGAA	CGTAACTTCCGGAAGAAGGA	162
IPO8	article[b]	EIE	NM_001206120.1	ENSBTAT00000018760	GCCCTTGCTCTTCAGTCATT	GTGCAACAGCTCCTGCATAA	93
KDM2B	MDAB[c]	EIE	XM_004017579.1	ENSOART00000015101	CGGTCCTACCTCACTCAGGA	CCGTCTATGCTGGGCTTTCT	74
LEP	article[b]	EIE	NM_173928.2	ENSBTAT00000019853	CCAGGATGACACCAAAACC	TGGACAAACTCAGGAGAGG	140
LHX8	MDAB[c]	EIE	XM_004003563.1	ENSOART00000013688	AGAGCACGCCACAAGAAACA	AGGGCTGGAGTCCAAGAGTT	199
NR3C1	article[a]	E	NM_001206634.1	ENSBTAT00000025941	ACTGCCCCAAGTGAAAACAGA	ATGAACAGAAATGGCAGACATTTTATT[j]	151
PGC1α	MDAB[d]	EE	NM_177945.3	ENSBTAT00000022636	GATTGGCGTCATTCAGGAGC	CCAGAGCAGCACACTCGAT	84
PGK1	MDAB[d]	EIE	NM_001034299.1	ENSBTAT00000001187	GCCTGTTGACTTTGTCACTGC	AGCAACAGCCTCAGCATACTT	142
PPARγ	article[b]	EIE	NM_181024.2	ENSBTAT00000044038	GACCCGATGGTTGCAGATTA	TGAGGGAGTTGGAAGGCTCT	145
PRDM16	MDAB[d]	EE[h]	XM_003583245.1	ENSBTAT00000025245	TGGCAGCTGGCTCAAGTACA	CGGAACGTGGGCTCCTCATC	198
PRLR	article[b]	EIE	NM_174155.3	ENSBTAT00000014437	CTCCACCCACCATGACTGAT	CAGCGAATCTGCACAAGGTA	169
RIP140	article[b]	E	XM_002684642.2	ENSBTAT00000065805	CGAGGACTTGAAACCAGAGC	TCTTAGGGACCATGCAAAGG	179

Gene	Source	Type	NCBI reference	Ensembl reference	Forward primer sequence	Reverse primer sequence	Length
RPLP0	article[f]	EIE	NM_001012682.1	ENSBTAT00000055086	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	227
SDHA	article[g]	EIE	NM_174178.2	ENSBTAT00000064827	CATCCACTACATGACGGAGCA	ATCTTGCCATCTTCAGTTCTGCTA	90
SHOX2	MP[d]	EIE	NM_001205527.1	ENSBTAT00000044731	CGCCTTTATGCGTGAAGAAC	TTGGCTGGCAGCTCCTAT	142
SREBF1	MDAB[c]	EE	XM_004013336.1	ENSOART00000001614	AGGGGGACAAGGAGTTCTCA	CTCCGGCCATATCCGAACAG	72
TBP	MP[d]	EIE	NM_001075742.1	ENSBTAT00000010109	CTTGGACTTCAAGATTCAGAACA	CCAGGAAATAACTCTGGCTCA	120
UCP1	MDAB[d]	EE[i]	XM_003587124.1	ENSBTAG00000004647	GGGCTTTGGAAAGGGACTACT	CAGGGCACATCGTCTGCTAAT	128
YWHAZ	MDAB[d]	EE	NM_174814.2	ENSBTAG00000000236	CCGGACACAGAACATCCAGTC	TCAGCTCCTTGCTCAGTTACAG	125

[a]   Williams *et al* [2007].

[b]   Pope *et al* [2014].

[c]   Designed to the sheep (*Ovis aries*) genome [Jiang *et al*, 2014].

[d]   Designed to the cow (*Bos taurus*) genome.

[e]   Sharkey *et al* [2009].

[f]   Robinson *et al* [2007].

[g]   Garcia-Crespo *et al* [2005].

[h]   One of the primers spans an exon-exon boundary (EE), but the primers also flank an intron (EIE).

[i]   Both the forward and reverse primers span an exon-exon boundary.

[j]   The cited article omits the last 5 bases from the reverse primer sequence.

2.4.10.5 Sequencing of products

The PCR products of primers whose sequences had not been previously published, or whose products had not been previously verified internally, were sent to the University of Nottingham School of Life Sciences DNA Sequencing Laboratory for analysis. The method used was high-sensitivity Sanger dideoxy sequencing [Sanger *et al*, 1977], and the process was carried out on an Applied Biosytems 3130*xL* Genetic Analyzer (Life Technologies). Both the forward and reverse primers were used for sequencing, to maximise confidence in the results. Sequences returned by the Sequencing Laboratory were verified by alignment with the predicted sequence using the online European Bioinformatics Institute (EBI) 'Clustal Omega' tool [EBI, 2014], and by 'blasting' against the appropriate genome(s) using the NCBI Standard Nucleotide BLAST tool [NCBI, 2014d] to ensure that they were specific to the intended target.

2.4.11 Preparation of serial dilutions for standard curves

For each gene tested, in order to assess the efficiency of qPCR reactions and/or to quantify the amount of cDNA in samples amplified by qPCR, a series of 1 in 10 dilutions was made from a known amount of cDNA. Initially, the known amount was a concentration of $1\,\mathrm{ng\,\mu l^{-1}}$ and dilutions were made in nuclease-free water. Later in the study, the known amount was 10^{10} cDNA copies and dilutions were made in a $10\,\mathrm{ng\,\mu l^{-1}}$ solution of yeast tRNA. In the latter case, the formula used to calculate the concentration required for 10^{10} copies was:

$$c = \frac{wln}{Av}$$
(2.12)

where:

c = concentration;
w = average molecular weight of a DNA molecule = $660\,\mathrm{g\,M^{-1}}$;
l = amplicon length (bp);
n = number of copies required = 10^{10};
A = Avogadro's constant = 6.02214×10^{23} molecules $\mathrm{M^{-1}}$;
v = reaction volume = $3\,\mathrm{\mu l}$.

Dilutions were prepared down to amounts of $10^{-10}\,\mathrm{ng\,\mu l^{-1}}$ or 10^1 copies, as appropriate.

2.4.12 qPCR

qPCR was carried out in 96-well microtitre plates. The reaction volume was 10 µl, comprising 5 µl Applied Biosystems Fast SYBR Green Master Mix (Life Technologies), 1 µl each of 2.5 µM forward and reverse primers, and 3 µl cDNA solution. Plates were covered with

adhesive seal, centrifuged for 1 min at 500 xg, and loaded into an Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies). Samples were heated to an initial temperature of 95 °C for 20 s to activate the DNA polymerase, then submitted to 40 cycles of melting at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Melt curves were produced for each sample by melting at 95 °C for 15 s, annealing/extension at 60 °C for 1 min, ramping the temperature up in 0.3 °C increments to 95 °C, and holding at 95 °C for 15 s.

An 8-point standard curve was produced for every plate, with points from 10^{-2}–10^{-9} ng μl^{-1} cDNA or 10^8–10^1 cDNA copy numbers. Serial dilutions for standard curves were run in triplicate: samples, calibrators and controls were run in duplicate. Only one gene was tested on each qPCR plate. For Study A, there were too many samples for a single plate, so two plates were used for each gene: 1 for the 7 day-old and the other for the 28 day-old samples. Five 1 day-old samples (used for the ontogeny study) were divided between the two plates. Two calibrator samples of perirenal adipose tissue from Study A animals were included on both Study A plates. All plates included 2–4 NRTCs, 1 NTC from the RT reaction and 1 NTC made up with 3 µl nuclease-free water in place of template. gDNA was run as a negative control on all Study B plates and on one from each pair of Study A plates, but it did not prove very useful, as it had a much lower reaction efficiency than the cDNA, and the results were difficult to interpret. Controls of muscle, 1 day-old perirenal fat (BAT) and 30 day-old perirenal fat (WAT) were used on the Study B plates. These served as either positive or negative controls, depending on the specific gene being tested. They were not used on the Study A plates because (1) there was insufficient room and (2) any problems with the primers would have been detected in Study B (processed earlier). There was one less sample of subcutaneous than sternal adipose tissue in Study B, so a duplicated RT reaction was carried out with mRNA from one of the samples of subcutaneous adipose tissue. This RT control was run to confirm that different RT reactions for the same sample would produce similar normalised qPCR results. The number of serial dilutions, samples, calibrators and controls for each tissue are summarised in Table 2.5, p 105.

For each study/tissue combination, the plate layout was the same for every gene tested. Although randomisation of the samples on each plate was considered, it was rejected for two reasons:

- a systematic layout was less prone to error;

- any temperature differences over the heating block of the qPCR machine would be controlled for, as the genes of interest and reference genes for each sample would be processed in the equivalent well.

Table 2.5 Loading of qPCR plates. The number of serial dilutions, samples, controls and calibrators is shown for each plate. Samples, controls and calibrators were run in duplicate; serial dilutions for standard curves were run in triplicate. All 96 wells of each plate were used. For Study A, two plates were used for each gene. Sternal is sternal adipose tissue and Subcut is subcutaneous adipose tissue.

Item	Study A		Study B	
	Sternal 1	Sternal 2	Sternal	Subcut
	No	No	No	No
Serial dilutions for standard curves	8	8	8	8
Control: muscle	-	-	1	1
Control: brown adipose tissue (BAT)	-	-	1	1
Control: white adipose tissue (WAT)	-	-	1	1
1 day-old samples	3	2	-	-
7 day-old samples	26	-	-	-
28 day-old samples	-	28	-	-
6 month-old samples	-	-	26	25
Reverse transcription (RT) control	-	-	-	1
Calibrators	2	2	-	-
Non-reverse transcriptase controls (NRTCs)	2	2	4	4
Non-template controls (NTCs) from RT reaction	1	1	1	1
NTC (nuclease free water)	1	1	1	1
Genomic DNA (gDNA)	1	-	1	1
Total	44	44	44	44

2.4.13 Selection of reference genes

As previously explained, the measured amount of mRNA for a particular gene in a sample depends not only on the total amount of mRNA, but also on RT and PCR reaction efficiency and other variables. It therefore needs to be 'normalised' so that different samples can be compared. This is usually achieved by reporting the ratios of the amounts of mRNA for genes of interest to those of one or more reference genes. A good reference gene should be stably and similarly expressed in all the tissues, experimental groups or developmental time points that are being compared in a particular study. Its abundance should also be closely correlated to the total amount of mRNA in a sample [Bustin *et al*, 2009]. In practice, the expression of all genes varies to some extent, so there is no perfect reference gene, and no single reference gene that fits all scenarios. It is therefore necessary to test potential reference genes for stability in the tissues, groups and time points under investigation. A number of candidate reference genes of varying cellular functionality were identified from previous experience in our laboratory, and by searching the literature. A total of eight such genes were tested for stability (Table 2.6, p 106). The candidate genes were tested with all samples from Study A and Study B, not just a subset, to maximise the reliability of

the results. A box and whisker plot of C_T values of candidate reference genes for the three tissues examined is shown in Figure 2.16, p 107.

A number of software packages have been developed to rank potential reference genes in order of stability from a panel of candidate genes (and in some cases to determine the optimum number to use for normalisation):

- geNorm [Vandesompele *et al*, 2002];

- NormFinder [Andersen *et al*, 2004];

- BestKeeper [Pfaffl *et al*, 2004].

All three packages use the qPCR results from a number of samples run with the candidate reference genes to make their selection. Two of the packages were used in the study, geNorm and NormFinder. They are both Microsoft Excel-based tools, though scripts were developed in R [R Core Team, 2014] from the published algorithms to automate the selection process and, in the case of NormFinder, to extend the analysis to groups of more than two genes.[27] All normalisation packages have to deal with the innate problem that the measured amounts of cDNA for candidate reference genes are not themselves normalised. The two packages deal with this problem in different ways. geNorm examines pairs of candidate genes under the assumption that if both are stably expressed, the ratio of their measured amounts of mRNA will be relatively constant, irrespective of the starting amount of cDNA. It tests all potential pairs from the panel of candidate genes. Its main disadvantage is that genes that are co-regulated, or whose expression is coincidentally correlated, will also have relatively constant expression ratios, even if they are not stably

Table 2.6 Candidate reference genes and their functions. The full name of each of the genes listed below is shown in the List of Abbreviations and Acronyms (p 15).

Gene	Function
18S	ribosomal RNA
IPO8	nuclear protein importer
KDM2B	histone demethylase, component of ubiquitin ligase complex
PGK1	glycolytic enzyme, DNA polymerase α cofactor
RPLP0	component of the ribosomal 60S subunit
SDHA	component of the mitochondrial electron transport chain complex II
TBP	transcription factor involved in expression of RNA polymerase II
YWHAZ	intracellular signalling mediator

[27] The creators of NormFinder have since developed their own R script. An error was found in their script (and in the algorithm published in the supplementary information to the original paper), which has now been amended and credited on the latest version [MOMA, 2015].
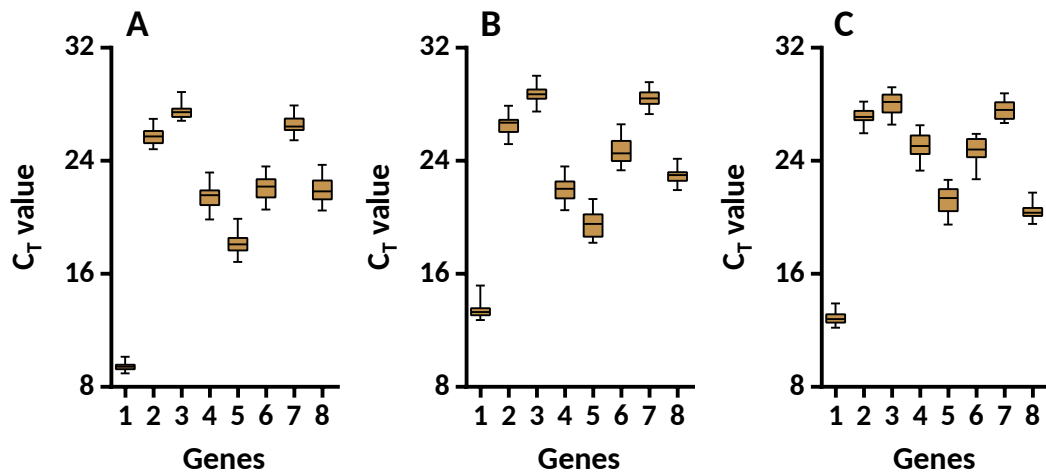
Figure 2.16 C_T values of candidate reference genes. Values shown are for Study A sternal fat (**A**), Study B sternal fat (**B**) and Study B subcutaneous fat (**C**). Boxes show the median and upper and lower quartiles, whiskers show the maximum and minimum values. The C_T values are not directly comparable, either within or between plots, because there is an element of subjectivity in placing the threshold line. However, the diagrams give an idea of the log transformed relative expression levels of each of the candidate reference genes and their spread. The genes are: 18S (1); IPO8 (2); KDM2B (3); PGK1 (4); RPLP0 (5); SDHA (6); TBP (7); and YWHAZ (8).

expressed. NormFinder is based on a mathematical model and makes the assumption that if the panel of candidate genes is sufficiently large, then the average expression of all the candidate genes will be the same. The expression of an individual candidate gene in a sample is therefore 'normalised' to the average expression of all candidate genes in that sample, and its relative stability over all samples can thereby be assessed. Unlike geNorm, NormFinder also has the capability to analyse the stability of potential reference genes across different experimental groups, and it calculates a stability value which is a function of both intra-group and inter-group variation.

The creators of geNorm recommend using the geometric mean expression of multiple reference genes for normalisation (a minimum of three), and the software produces a figure for the 'optimum' number of reference genes. However, the criterion for this is rather arbitrary, and the multiple genes selected are simply based on individual rankings, with no facility to examine the relative stability of gene combinations. In contrast, NormFinder does not recommend an optimum number of genes, but does allow stability values to be calculated for combinations (and thereby compared to single genes). This is considered a more robust methodology, because a combination of the two most stable individual genes will not necessarily be an improvement over a single gene (eg if the inter-group variation for both genes is in the same direction).

Because of their different underlying assumptions, if both methods suggested the same reference genes, there could be a high degree of confidence in the selection. In contrast, if the two methods suggested different genes, the decision was more subjective. In the latter

case, more weight was given to NormFinder because of its ability to analyse stability across experimental groups and to evaluate combinations of genes. In the event, the selection of reference genes for Study A and Study B sternal adipose tissue proved relatively straight-forward, while the selection for Study B subcutaneous tissue was more difficult.

The strategy with Study A was to find a gene, or combination of genes, which was stably expressed both within and between the two age groups. A secondary consideration was stability within the individual sexes. Only one grouping variable, feeding groups, was used with NormFinder. The ranking of individual reference genes as determined by geNorm and NormFinder for Study A is shown in Table 2.7, p 109. There was reasonably good correlation between geNorm and NormFinder both within and between age groups. Three of the candidate genes, 18S, SDHA and PGK1, could be eliminated immediately, as they were ranked poorly under both methods and across both age groups. YWHAZ was also rejected because, although it was reasonably stable within age groups, it was the lowest ranked gene when the 7- and 28-day groups were considered together. KDM2B showed the largest variation between the two methods: while it was ranked quite highly by both methods across age groups, and within age groups by NormFinder, it was consistently ranked sixth out of eight within age groups by geNorm. It was therefore initially decided to reject it. Of the three remaining genes, IPO8, RPLP0 and TBP, the three-gene combination was better than any single gene or two-gene combination across the age groups and within the 28-day groups, though within the 7-day group it was less stable than the best single gene and two-gene combination (Table 2.8, p 109). It was therefore decided to reintroduce KDM2B, as it appeared in some of the best combinations. The four-gene combination of IPO8, KDM2B, RPLP0 and TBP was an improvement on the three-gene combination of IPO8, RPLP0 and TBP in every case, including individual sexes (not shown), and though there were better two- and three-gene combinations in the 7-day group, the stability value of the four-gene combination (0.0602) was only marginally inferior to that of the best option (0.0551). The geometric mean expression of IPO8, KDM2B, RPLP0 and TBP was therefore used to normal-ise the results for Study A sternal adipose tissue.

With Study B, no calibrator was included on the qPCR plates, so no direct comparison would have been possible between sternal and subcutaneous adipose tissue. The strategy, therefore, was to find the most stable reference gene, or combination of reference genes, in each of the two tissues, notwithstanding that these might be different. The grouping capability of NormFinder was used to find a single gene or combination of genes which was stable across both prenatal and postnatal groups. The ranking of individual genes by geNorm and NormFinder for Study B sternal adipose tissue is shown in Table 2.9, p 110. There was a reasonably good correlation between the geNorm results and those of both

Table 2.7 Study A sternal adipose tissue: ranking of potential reference genes. Genes were tested by third party software geNorm (gN) and NormFinder (NF), in the latter case testing for stability across feeding groups. Genes were tested both within and between age groups (7 and 28 days). M is male and F is female. A combination of IPO8, KDM2B, RPLP0 and TBP was ultimately selected for normalisation.

Test	Age	Sex	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5	Rank 6	Rank 7	Rank 8
gN	7/28	All	IPO8	TBP	RPLP0	KDM2B	18S	SDHA	PGK1	YWHAZ
		M	IPO8	TBP	KDM2B	18S	RPLP0	SDHA	YWHAZ	PGK1
		F	IPO8	TBP	RPLP0	KDM2B	18S	SDHA	PGK1	YWHAZ
	7	All	IPO8	YWHAZ	TBP	RPLP0	SDHA	KDM2B	18S	PGK1
		M	YWHAZ	TBP	IPO8	RPLP0	SDHA	KDM2B	18S	PGK1
		F	IPO8	YWHAZ	TBP	RPLP0	SDHA	KDM2B	18S	PGK1
	28	All	TBP	IPO8	RPLP0	YWHAZ	SDHA	KDM2B	18S	PGK1
		M	IPO8	RPLP0	TBP	YWHAZ	SDHA	KDM2B	18S	PGK1
		F	TBP	RPLP0	IPO8	YWHAZ	SDHA	KDM2B	18S	PGK1
NF	7/28	All	IPO8	KDM2B	RPLP0	TBP	18S	SDHA	PGK1	YWHAZ
		M	IPO8	KDM2B	TBP	18S	RPLP0	SDHA	PGK1	YWHAZ
		F	IPO8	KDM2B	RPLP0	TBP	18S	SDHA	PGK1	YWHAZ
	7	All	IPO8	KDM2B	RPLP0	YWHAZ	TBP	18S	SDHA	PGK1
		M	IPO8	TBP	YWHAZ	RPLP0	KDM2B	SDHA	18S	PGK1
		F	KDM2B	IPO8	RPLP0	TBP	YWHAZ	18S	SDHA	PGK1
	28	All	RPLP0	IPO8	TBP	SDHA	KDM2B	YWHAZ	18S	PGK1
		M	RPLP0	SDHA	IPO8	TBP	KDM2B	YWHAZ	18S	PGK1
		F	RPLP0	TBP	KDM2B	IPO8	SDHA	18S	PGK1	YWHAZ

Table 2.8 Study A sternal adipose tissue: selected stability values. Combinations of potential reference genes were tested by NormFinder software for stability across feeding groups both within and between age groups (7 and 28 days). The best single gene and multiple-gene combinations in each category, plus other selected combinations, are shown. SV is stability value. The lowest value indicates the most stable gene or combination. A combination of IPO8, KDM2B, RPLP0 and TBP was ultimately selected for normalisation.

Age	Item	Gene(s)	SV
7/28	Best single gene	IPO8	0.0450
	Best two gene combination	IPO8/KDM2B	0.0416
	Best three gene combination	IPO8/KDM2B/RPLP0	0.0385
	Other three gene combination	IPO8/RPLP0/TBP	0.0407
	Four gene combination	IPO8/KDM2B/RPLP0/TBP	0.0352
7	Best single gene	IPO8	0.0734
	Best two gene combination	IPO8/KDM2B	0.0595
	Best three gene combination	IPO8/KDM2B/RPLP0	0.0551
	Other three gene combination	IPO8/RPLP0/TBP	0.0747
	Four gene combination	IPO8/KDM2B/RPLP0/TBP	0.0602
28	Best single gene	RPLP0	0.0855
	Best two gene combination	IPO8/RPLP0	0.0624
	Best three gene combination	IPO8/RPLP0/TBP	0.0528
	Four gene combination	IPO8/KDM2B/RPLP0/TBP	0.0492

the NormFinder tests. All three tests ranked TBP and IPO8 as the best two genes overall, with some combination of YWHAZ and KDM2B as the third and fourth best (though there was some variation between sexes). Selected NormFinder stability values for various genes and combinations are shown in Table 2.10, below. IPO8, TBP and YWHAZ was the most stable three-gene combination across both postnatal and prenatal groups, and it was more stable than the best single gene or two-gene combination in each case. Three was also the optimum number of genes recommended by geNorm. The geometric mean expression of IPO8, TBP and YWHAZ was therefore used to normalise the results for Study B sternal adipose tissue.

Table 2.9 Study B sternal adipose tissue: ranking of potential reference genes. Genes were tested by third party software geNorm (gN) and NormFinder (NF), in the latter case testing stability across both postnatal and prenatal feeding groups. M is male and F is female. NA is not applicable. A combination of IPO8, TBP and YWHAZ was ultimately selected for normalisation.

Test	Groups	Sex	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5	Rank 6	Rank 7	Rank 8
gN	NA	All	TBP	IPO8	YWHAZ	KDM2B	RPLP0	SDHA	PGK1	18S
		M	TBP	YWHAZ	IPO8	KDM2B	RPLP0	SDHA	PGK1	18S
		F	TBP	IPO8	PGK1	YWHAZ	KDM2B	SDHA	18S	RPLP0
NF	Postnatal	All	TBP	IPO8	KDM2B	YWHAZ	SDHA	PGK1	18S	RPLP0
		M	IPO8	TBP	KDM2B	YWHAZ	SDHA	PGK1	RPLP0	18S
		F	TBP	IPO8	18S	KDM2B	SDHA	PGK1	YWHAZ	RPLP0
	Prenatal	All	TBP	IPO8	YWHAZ	KDM2B	SDHA	PGK1	RPLP0	18S
		M	IPO8	YWHAZ	TBP	KDM2B	SDHA	PGK1	RPLP0	18S
		F	TBP	IPO8	KDM2B	PGK1	SDHA	18S	YWHAZ	RPLP0

Table 2.10 Study B sternal adipose tissue: selected stability values. Combinations of potential reference genes were tested by NormFinder software for stability across postnatal and prenatal feeding groups. The best single gene and multiple-gene combinations in each category (postnatal and prenatal) are shown. SV is stability value. The lowest value indicates the most stable gene or combination. A combination of IPO8, TBP and YWHAZ was ultimately selected for normalisation.

Groups	Item	Gene(s)	SV
Postnatal	Best single gene	TBP	0.1405
	Best two gene combination	KDM2B/TBP	0.0857
	Best three gene combination	IPO8/TBP/YWHAZ	0.0566
	Best four gene combination	KDM2B/SDHA/TBP/YWHAZ	0.0607
Prenatal	Best single gene	TBP	0.0672
	Best two gene combination	IPO8/TBP	0.0485
	Best three gene combination	IPO8/TBP/YWHAZ	0.0463
	Best four gene combination	IPO8/KDM2B/TBP/YWHAZ	0.0461

The ranking of individual genes by geNorm and NormFinder for Study B subcutaneous adipose tissue is shown in Table 2.11, below. In this case there was very poor correlation between the geNorm results and those of each of the NormFinder tests, other than the fact that 18S and KDM2B were the lowest ranked genes on all three tests. geNorm ranked SDHA, PGK1 and RPLP0 as the best three genes, with considerable variation between the sexes. NormFinder showed some correlation to geNorm on the postnatal test, with TBP, SDHA and PGK1 ranked as the best three genes. However, the results of the NormFinder test across prenatal groups was more akin to those of sternal adipose tissue, with IPO8, TBP and RPLP0 ranked as the best three genes. Selected NormFinder stability values for various genes and combinations are shown in Table 2.12, p 112. The stability values and rankings of the various combinations were considerably different between the two tests, so ultimately a compromise had to be reached. The four-gene combination of PGK1, SDHA, TBP and YWHAZ had the lowest (ie best) average stability value of any single genes or combinations over both tests, and the stability value was similar for both (0.0641 for postnatal and 0.0644 for prenatal). The geometric mean expression of SDHA, PGK1, TBP and YWHAZ was therefore used to normalise the results for Study B subcutaneous adipose tissue.

2.4.14 Analysis of results

The results from Study B were quantified using the Pfaffl method of relative quantification (Equation 2.10, p 87). The calibrator groups were the respective control groups for both prenatal and postnatal analysis. Efficiencies were calculated from the slope of the standard curve produced from the same qPCR run as the gene being quantified (Equation 2.8, p 84).

Table 2.11 Study B subcutaneous adipose tissue: ranking of potential reference genes. Genes were tested by third party software geNorm (gN) and NormFinder (NF), in the latter case testing stability across both postnatal and prenatal feeding groups. M is male and F is female. NA is not applicable. A combination of PGK1, SDHA, TBP and YWHAZ was ultimately selected for normalisation.

Test	Groups	Sex	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5	Rank 6	Rank 7	Rank 8
gN	NA	All	SDHA	PGK1	RPLP0	IPO8	TBP	YWHAZ	KDM2B	18S
		M	TBP	IPO8	YWHAZ	PGK1	SDHA	RPLP0	KDM2B	18S
		F	PGK1	SDHA	RPLP0	IPO8	TBP	YWHAZ	KDM2B	18S
NF	Postnatal	All	TBP	SDHA	PGK1	YWHAZ	IPO8	RPLP0	18S	KDM2B
		M	IPO8	TBP	SDHA	PGK1	YWHAZ	RPLP0	KDM2B	18S
		F	YWHAZ	TBP	SDHA	IPO8	PGK1	RPLP0	18S	KDM2B
	Prenatal	All	IPO8	TBP	RPLP0	YWHAZ	SDHA	PGK1	18S	KDM2B
		M	IPO8	TBP	SDHA	YWHAZ	PGK1	KDM2B	RPLP0	18S
		F	TBP	IPO8	YWHAZ	PGK1	RPLP0	SDHA	KDM2B	18S

Table 2.12 Study B subcutaneous adipose tissue: selected stability values. Combinations of potential reference genes were tested by NormFinder software for stability across postnatal and prenatal feeding groups. The best single gene and multiple-gene combinations in each category (postnatal and prenatal) are shown, together with the corresponding best combinations from the other category. SV is stability value. The lowest value indicates the most stable gene or combination. A combination of PGK1, SDHA, TBP and YWHAZ was ultimately selected for normalisation.

Groups	Item	Gene(s)	SV
Postnatal	Best single gene	TBP	0.1379
	Best two gene combination	TBP/YWHAZ	0.0889
	Other two gene combination	IPO8/TBP	0.1541
	Best three gene combination	SDHA/TBP/YWHAZ	0.0736
	Other three gene combination	IPO8/TBP/YWHAZ	0.1079
	Best four gene combination	PGK1/SDHA/TBP/YWHAZ	0.0641
	Other four gene combination	IPO8/RPLP0/TBP/YWHAZ	0.1260
Prenatal	Best single gene	IPO8	0.0838
	Best two gene combination	IPO8/TBP	0.0651
	Other two gene combination	TBP/YWHAZ	0.0818
	Best three gene combination	IPO8/TBP/YWHAZ	0.0616
	Other three gene combination	SDHA/TBP/YWHAZ	0.0713
	Best four gene combination	IPO8/RPLP0/TBP/YWHAZ	0.0575
	Other four gene combination	PGK1/SDHA/TBP/YWHAZ	0.0644

In the early part of the study, standard curves were occasionally poor or incomplete. In that event, efficiencies calculated from standard curves produced from other qPCR runs with the same gene/primer combination were used instead. While this may have introduced some error, it was considered a better approximation than the $2^{-\Delta\Delta C_T}$ method.

In contrast, absolute quantification was used in Study A, and the results are reported in terms of normalised DNA copy number. The 'best' standard curve from each pair of plates, usually either the one with the coefficient of determination (r^2) closest to 1 or the one with the tightest replicates at low copy number, was used for quantification. The C_T values of samples on the same plate as the selected standard curve (the 'quantification plate') could be used directly to determine copy number. From Equation 2.5, p 84:

$$\log N_0 = m C_T + c \tag{2.13}$$

where:

N_0 = initial number of molecules;
m = slope of the standard curve;
C_T = the threshold cycle;
c = y-axis intercept of the standard curve.

The same formula was used for quantification of samples on the other plate, but the C_T values were first adjusted using the C_T values of the calibrators:

$$C_{T_{adjusted}} = C_{T_{original}} + C_{T_{calibs\,(quant)}} - C_{T_{calibs\,(other)}} \qquad (2.14)$$

where:

$C_{T_{adjusted}}$ = sample adjusted C_T value;

$C_{T_{original}}$ = sample original C_T value;

$C_{T_{calibs\,(quant)}}$ = mean C_T value of calibrators on the quantification plate;

$C_{T_{calibs\,(other)}}$ = mean C_T value of calibrators on the other plate.

If replicates were poor for one of the two calibrators, then only one was used. If they were poor for both calibrators, then the plate was rejected and repeated.

C_T values were exported directly from the qPCR machine to a Microsoft Excel spreadsheet. In order to remove the possibility of error and/or distortion of data arising from the manipulation (particularly 'copy and paste' operations) of a large number of spreadsheets (more than 100), scripts were written in R [R Core Team, 2014] to automate the calibration, quantification and normalisation processes and, for Study A, the production of standard curves. A copy of each spreadsheet was saved as a comma-separated variable (CSV) file, non-essential rows and columns were deleted,[28] and additional columns were inserted to indicate the plate number, date and (for Study B) the reaction efficiency (which was manually recorded from the qPCR machine). The R scripts read in the data from the CSV files for subsequent processing, but the files themselves were never subsequently amended or overwritten. All results and other reports were written to newly-created CSV files.

2.5 Protein expression

2.5.1 Introduction

UCP1 protein expression was determined for Study A sternal adipose tissue samples from 7 day-old lambs. Samples were lysed and homogenised, mitochondria were separated out by a process of differential centrifugation, and total mitochondrial protein concentration was measured. Preparations were then diluted such that they all contained the same quantity of protein, and the relative amount of UCP1 in each sample was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. There was insufficient tissue available to perform any protein expression work on Study B tissues.

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[28] The quantification threshold column was retained, so that C_T values could always be reproduced from the raw qPCR machine data file if necessary.

2.5.2 Principle of the assay

2.5.2.1 Principle of SDS-PAGE

SDS-PAGE is a technique that uses electrophoresis to separate denatured proteins based on their molecular weight [Shapiro et al, 1967]. The medium used for electrophoresis is a polyacrylamide gel, which is porous and chemically inert. It is formed by the polymerisation of acrylamide with a small amount of bisacrylamide, producing a highly cross-linked 3-dimensional matrix whose pore size is determined by the concentration and relative quantities of the two constituents. For a specific strength of electric field, a molecule's speed of migration through a particular gel will depend on its size, shape and charge. In order to separate proteins based on their molecular weight, therefore, their charge and shape must be standardised. This is largely achieved by treatment with a negatively-charged ionic detergent, sodium dodecyl sulfate (SDS), which binds to proteins at a ratio of about one SDS ion to every two amino acids. Proteins may have either positive or negative charges, depending on the composition of their charged amino acids, but their intrinsic charge is insignificant compared to the high negative charge of the bound SDS ions. The charge of a protein-SDS complex is therefore approximately proportional to its length, and thus its mass (assuming an average mix of amino acids). When an electric field is applied, therefore, proteins will migrate through the gel towards the positive electrode. SDS also removes most of the secondary and tertiary structure of proteins by breaking down the non-covalent molecular interactions involved in protein folding, leaving only covalent disulfide (S–S) bonds. These bonds can be reduced by treatment with 3-mercaptoethanol or dithiothreitol, without affecting stronger covalent bonds such as C–C or C–H, to give a fully denatured, linear protein chain. Denatured proteins of the same size migrate through the gel at roughly the same speed, as they have the same shape and charge. Small proteins migrate more quickly, while larger ones migrate more slowly as the effect of greater drag outweighs their higher charge. Proteins are therefore separated into a series of discrete protein bands based on their molecular weight [Alberts et al, 2008; Berg et al, 2012].

2.5.2.2 Principle of western blotting

Once proteins have been separated on a gel, specific proteins can be detected by immuno-assay via a technique termed 'western blotting' [Burnette, 1981; Towbin et al, 1979].[29] The

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[29] The term 'western blotting' is a play on words [Burnette, 1981]. The original technique for the transfer of DNA to a membrane was developed by Edwin Southern, and is therefore known as 'Southern blotting' [Southern, 1975]. The equivalent techniques for RNA and proteins were termed 'northern' and 'western' blotting respectively.

proteins are first transferred or 'blotted' onto a nylon, nitrocellulose or polyvinylidene difluoride membrane, which makes them more accessible for subsequent reactions. The method of transfer is either capillary action ('wet' blotting) or electrophoresis (which can be either 'wet', 'semi-dry' or 'dry'). In this study, an electrophoretic wet blotting system was used, whereby the membrane and gel are sandwiched together, immersed in a tank of transfer buffer, and subjected to an electric field. The proteins are transferred in the same pattern as the gel, and can be visualised by staining the membrane with a dye. This gives simple confirmation of effective protein transfer and consistent protein loading. A protein of interest on a membrane can be detected by immunolabelling, a technique which uses antibodies linked to a detectable 'tag'. Antibodies are proteins produced by an immune system as a defence against infection or harmful substances, and each antibody binds only to a specific target molecule or 'antigen'. An antigen induces the formation of an antibody and has one or more antibody-binding sites, highly-specific regions on the surface of the molecule that are termed 'epitopes'. Antibodies can be 'polyclonal', where they target all the epitopes on a particular antigen, or 'monoclonal', where they target a single epitope (Figure 2.17, p 116). Specific antibodies are obtained by injecting the antigen (ie the protein of interest) into an animal from another species, allowing time for antibodies against that antigen to develop, then collecting them from the serum. The membrane is first 'blocked' with a non-relevant protein (usually milk or bovine serum albumin (BSA)) to prevent non-specific binding of antibodies. It is then exposed to an antibody specific to the protein of interest (Figure 2.18, p 117). While a tag can be directly linked to this 'primary' antibody ('direct detection'), the detectable signal can be amplified if a two-stage process is used ('indirect detection'). A membrane that has been treated with an unlabelled primary antibody is exposed to a labelled 'secondary' antibody (raised in a different species of animal) that is specific to the primary antibody. Amplification is achieved because the secondary antibody can bind to different epitopes on the primary antibody. A tag is covalently linked to an antibody, and is radioactive, fluorescent or enzymatic. An enzymatic tag produces a detectable signal, such as a coloured precipitate or chemiluminescence, on treatment with a substrate [Alberts *et al*, 2008; Berg *et al*, 2012]. Chemiluminescence is the emission of light as a result of a chemical reaction. Fluorescence and chemiluminescence can be detected with X-ray film or charge-coupled device (CCD) cameras [Alegria-Schaffer *et al*, 2009]. In this study, the secondary antibody was labelled with an enzyme, horseradish peroxidase (HRP), which oxidises the substrate luminol, causing the emission, in combination with other reagents, of enhanced chemiluminescence (ECL). ECL is a phenomenon whereby the addition of certain phenol derivatives increases the intensity of the light emission from chemiluminescence by more than 1 000-fold [Thorpe *et al*, 1985].
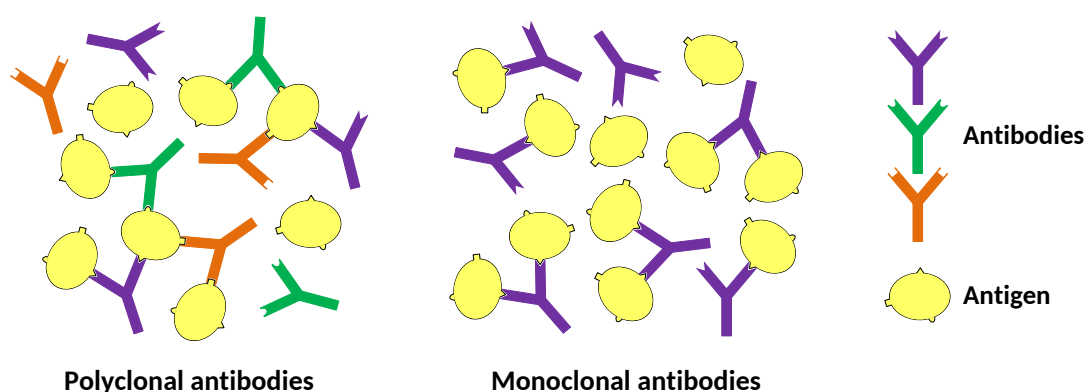
Figure 2.17 Polyclonal and monoclonal antibodies. Most antigens have several antibody-binding sites or 'epitopes'. Polyclonal antibodies are heterogeneous mixtures of antibodies, each of which is specific to one epitope on an antigen. Monoclonal antibodies are identical, and bind to one specific epitope (adapted from Berg *et al* [2012]).

2.5.3 Mitochondrial preparation

Three stock solutions were prepared: 250 ml of 1 M tris (tris(hydroxymethyl)methylamine, molecular weight (mw) 121.14, BDH, GPR; VWR, Lutterworth, Leicestershire, UK), pH 6.8; 1 l of 1 M sucrose (mw 342.3; Fisher Scientific); 500 ml of 0.5 M ethylenediaminetetraacetate (EDTA; disodium EDTA dihydrate, mw 372.24, BDH, AnalaR; VWR), pH 8.0. A 1 l stock of mitochondrial homogenisation buffer (MHB), pH 7.4, was made up from 10 ml 1 M tris solution (10 mM), 250 ml 1 M sucrose solution (250 mM) and 2 ml 0.5 M EDTA solution (1 mM), and filter sterilised with a water vacuum. All solutions and the MHB were made up from distilled water, and the pH was adjusted with concentrated hydrochloric acid (Fisher Scientific). Approximately 200 mg frozen tissue from each sample was chopped, weighed and placed in a gentleMACS tissue dissociation tube, to which 1 ml MHB was added. The chopped tissue was allowed to thaw on ice and then homogenised with a Dispomix drive. The tubes were centrifuged at 3 000 xg for 1 min at 4 °C, and the homogenate drawn off into 1.5 ml Eppendorf tubes. The tissue was further homogenised using a hand-held Potter-Elvehjem pestle (10 strokes), and the tubes of homogenate were then centrifuged at 800 xg for 10 min at 4 °C (to concentrate nuclei and cellular debris at the bottom of each tube and fat at the top). The supernatant was drawn off into a new 1.5 ml Eppendorf tube, and any remaining fat was carefully removed with a cotton bud. The tubes of supernatant were then centrifuged at 13 000 xg for 1 h at 4 °C, after which the supernatant was drawn off, leaving a mitochondrial pellet at the bottom of each tube. The pellets were resuspended by adding 100 µl MHB to each tube and pipette mixing. The suspensions were stored at –20 °C.
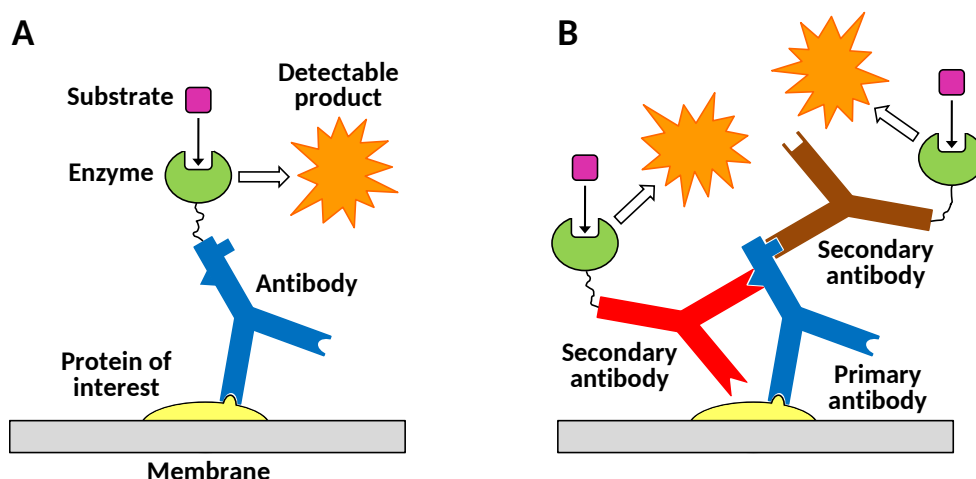
Figure 2.18 Immunodetection with labelled antibodies. In direct detection (**A**), a detectable 'tag' or 'label' is covalently linked to an antibody specific to the protein of interest. Indirect detection (**B**) uses an unlabelled 'primary' antibody specific to the protein of interest, followed by a labelled 'secondary' antibody specific to the primary antibody. This facilitates amplification of the signal. The tags can be radioactive, fluorescent or, as shown here, enzymatic. When a substrate binds to the enzyme, a detectable signal such as a coloured precipitate or chemoluminescence is produced.

2.5.4 Determination of protein concentration by BCA assay

Protein concentration is usually obtained by comparing the assay response of an unknown sample to those of a set of standards of known concentration. Samples and standards are processed in the same manner, and their absorbances are measured by spectrophotometer [Thermo Scientific, 2007]. The total protein concentration of the mitochondrial preparations was determined by the bicinchoninic acid (BCA) assay, which is based on a reaction between protein, copper sulfate and BCA [Smith *et al*, 1985]. In alkaline solution, copper sulfate and BCA form a green-coloured complex. However, the peptide bonds of proteins reduce Cu^{2+} ions to Cu^{+} ions (the 'biuret reaction'), and the latter form an intense purple complex with BCA, with an absorbance maximum at 562 nm.

Three reagents were prepared for the assay: Reagent A contained 0.5 g sodium bicinchoninate, 1 g sodium carbonate (anhydrous, BDH, AnalaR; VWR), 0.08 g sodium tartrate (dihydrate), 0.2 g sodium hydroxide (Fisher Scientific) and 0.5 g sodium bicarbonate, made up to a total volume of 50 ml with distilled water, and pH adjusted to 11.25 with 10 % sodium bicarbonate; Reagent B contained 2 g copper sulfate (pentahydrate; Fisher Scientific) made up to a total volume of 50 ml with distilled water; Reagent C, a clear green solution, contained a mixture of Reagent A and Reagent B in a 100:2 ratio by volume. All reagents were stored at 4 °C for up to a week. A volume of 10 μl of each sample was diluted 1 in 10 in 0.9 % saline to make up a volume of 100 μl. Concentrations of 1.0, 0.8, 0.6, 0.4, 0.3,

0.2 and 0.1 mg ml^{-1} BSA in 0.9 % saline were made up in a volume of 100 μl as standards. An amount of 10 μl of each standard, sample or negative control (0.9 % saline) was dispensed in triplicate into a 96-well microtitre plate, and 200 μl Reagent C was added to each well. The plate was covered with clingfilm, incubated on a shaker (Stuart Scientific Orbital Incubator SI50; Bibby Scientific Ltd, Stone, Staffordshire, UK) at 37 °C for 30 min for purple colour development, then allowed to cool to room temperature. The absorbance was read at 570 nm on a μQuant Universal Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

A best-fit standard curve was plotted from the absorbances of the standard concentrations. The relationship between absorbance and protein concentration is curvilinear rather than linear [Thermo Scientific, 2007], so a quadratic relationship was assumed in deriving the standard curve. All standard curves plotted on this basis had a coefficient of determination (r^2) in excess of 0.998. The protein concentration for each sample was calculated by inserting its absorbance into the quadratic equation for the standard curve. The assay was repeated for samples where the coefficient of variation (CV) between replicates was greater than 5 %. After these repeats, all but one sample had a CV of less than 6 %. The single exception was the sample with the highest measured concentration, and for this sample the average concentration from the two assays was used. A 10X multiplication factor was applied to the measured concentrations to account for the initial dilution.

2.5.5 Sample preparation

A stock of protein dissociation buffer (PDB) was prepared containing 50 mM tris (final concentration), 10 % glycerol, 2 % SDS, 5 % 3-mercaptoethanol and distilled water, with the pH adjusted to 6.8. A stock of bromophenol blue solution was prepared containing 0.01 g bromophenol blue (sodium salt), 14.9 ml sodium hydroxide and 23.5 ml distilled water. A solution of running dye (glycerol-bromophenol blue) was then made up containing 400 μl stock bromophenol blue solution, 800 μl glycerol and 3.8 ml distilled water. The protein samples from mitochondrial preparations were diluted with MHB to a concentration of 1.5 mg ml^{-1} in a volume of 40 μl, and then diluted further with 100 μl PDB and 28 μl running dye to a concentration of 0.357 mg ml^{-1} in a volume of 168 μl. One sample from an unused mitochondrial preparation was selected as a calibrator to be run on all gels. A negative control for UCP1 was prepared, containing 40 μl liver mitochondrial protein solution, 100 μl PDB and 28 μl running dye. The tubes of samples, calibrator and negative control were inserted into a bath of water at 95–100 °C and incubated for 10 min to denature the protein. Samples were stored at –20 °C pending electrophoresis.

2.5.6 SDS-PAGE

Gel preparation and electrophoresis were carried out using a Mini-PROTEAN 3 Cell kit (Bio-Rad, Hercules, CA, USA). The gel preparation part of the kit comprises a casting stand, which can hold two gels, 4 glass plates (a 'spacer plate' and a 'short plate' for each gel), 2 casting frames (which hold the spacer plates and short plates together) and 2 rubber seals. All gels were run in duplicate. Sufficient mixture for 2 × 0.75 mm 12 % polyacrylamide resolving gels was prepared, containing 3.3 ml distilled water, 4 ml 30 % polyacrylamide mix (acrylamide/bisacrylamide 37.5:1), 2.5 ml tris (1.5 M, pH 8.8), 100 µl 10 % SDS, 100 µl 10 % ammonium persulfate (APS) and 4 µl N,N,N',N'-tetramethylethylenediamine (TEMED). The apparatus was assembled (ie the glass plates were inserted into the casting frames and the casting frames were placed on the rubber seals and clipped to the casting block), and the resolving gel mixture was dispensed between the two glass plates of each casting frame with a 10 ml syringe, leaving a gap at the top of some 3 cm for stacking gel. Some water-saturated butanol (Fisher Scientific) was then poured onto the top of each gel, to level it and stop it from drying out. The resolving gel was left to set for 45 min, after which the butanol was poured off, and the top of the gel washed with distilled water. Sufficient mixture for two stacking gels was prepared, containing 3.4 ml distilled water, 850 µl 30 % polyacrylamide mix, 625 µl tris (1 M, pH 6.8), 50 µl 10 % SDS, 50 µl 10 % APS and 5 µl TEMED. The stacking gel mixture was dispensed onto the top of each resolving gel with a 10 µl syringe, a plastic comb was inserted into the stacking gel between each pair of glass plates, and the gel was left to set for 15 min.

The electrophoresis part of the kit comprises an electrophoresis tank, a lid with electric terminals, a clamping frame and an electrode assembly. When the stacking gel had set, the casting frames were unclipped from the casting stand, each pair of glass plates enclosing a polymerised gel (a 'gel cassette sandwich') was carefully removed from its casting frame and inserted into the electrode assembly (with the short plate facing inwards), the electrode assembly and two gel cassette sandwiches were inserted into the clamping frame, and the clamping frame was lowered into the tank. A stock of 5X running buffer was prepared, containing 15.1 g tris, 94 g glycine (Fisher Scientific) and 50 ml 10 % SDS solution per litre, with the balance made up with distilled water. The central chamber between the two gel cassette sandwiches was filled, and the bottom of the tank partially filled (to a depth of about 3 cm), with 1X running buffer (5X running buffer diluted 1 in 5 with distilled water). The plastic combs were removed from the stacking gel, the wells were rinsed with 1X running buffer using a 10 ml syringe, and the samples were loaded using a 50 µl pipette and long tips. Unused wells on the outside of the gel were loaded with 28 µl run-

ning dye solution (MHB, PDB and running dye in the proportions 40:100:28) to ensure even running. The wells adjacent to the running dye lanes were loaded with 10 μl protein ladder (PageRuler Plus Prestained Protein Ladder; Thermo Pierce, Rockford, IL, USA), the middle well with 10 μl negative control, one well with 28 μl calibrator, and the remaining wells with 28 μl sample. A volume of 28 μl at a concentration of 0.357 mg ml^{-1} (Section 2.5.5, p 118) equates to 10 μg protein. Allocation of samples to lanes was randomised with the Microsoft Excel RAND function. The lid was placed on the tank and connected to a power source, and electrophoresis was run at 200 V for 25 min (or until the dye front reached the bottom of the plates).

2.5.7 Western blotting

Following SDS-PAGE, the power supply was disconnected from the electrophoresis tank, the running buffer was poured off, and the apparatus was dismantled. For each gel cassette sandwich, an 'X' was inscribed on the top left of the large glass plate and the top right of the short glass plate with a marker pen. The two plates were carefully prised apart, leaving the gel adhered to one of them. The gel and adhering plate combination were laid flat with the gel on top, and the stacking gel was carefully removed with a spatula. The top corner of the resolving gel corresponding to the 'X' marked on the adhering glass plate was then cut off (to orientate the gel, by marking its top left as viewed from the front when the samples were loaded). Proteins were transferred from gels to a nitrocellulose membrane using a water-cooled electrophoretic transfer cell (Bio-Rad). The apparatus comprises an electrophoresis tank with a separate lower chamber containing inputs and outputs for water cooling, a lid with electrical terminals, and loading cassettes for the gel-membrane stack. A stock of 2.5 l transfer buffer was prepared, containing 14.5 g tris, 7.25 g glycine, 9.25 ml 10 % SDS solution and 500 ml methanol, with the balance made up with distilled water. For each gel, one piece of nitrocellulose membrane (Amersham Hybond ECL Nitro-cellulose Membrane; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and 4 pieces of Whatman paper (GE Healthcare) were cut out and soaked in transfer buffer. A stack was prepared consisting of two pieces of Whatman paper, the nitrocellulose membrane, the resolving gel, and the remaining two pieces of Whatman paper. The paper and membrane were trimmed, and the stack was gently rolled to remove air bubbles. A scouring pad was placed on the bottom and top of the stack, the stack was placed in the loading cassette between two sponges, and the cassette was inserted into the tank such that the membrane was located between the gel and the positive electrode (so that the negatively-charged proteins would be transferred onto the membrane). When all cassettes had been inserted, the tank was filled with transfer buffer, the lid was attached and connected to a power

source, the water supply was connected and turned on, and electrophoresis was run at 25 V overnight.

2.5.8 Protein staining

Membranes were stained with Ponceau S to provide visual confirmation of protein transfer and even loading. Ponceau S, a red dye, binds reversibly to proteins and reveals multiple horizontal bands in vertical lanes on a blotted membrane, corresponding to each well of the resolving gel. A 100 ml stock of Ponceau S stain was prepared, containing 2 g Ponceau S (sodium salt), 30 g trichloroacetic acid (Fisher Scientific) and 30 g sulfosalicylic acid (Acros Organics, dihydrate; Fisher Scientific), with the balance made up with distilled water. A 5 l stock of tris-buffered saline (TBS) was prepared, containing 12.1 g tris and 146.1 g sodium chloride, with the balance made up with distilled water and the pH adjusted to 7.5. A 1 l stock of TBS with Tween 20 (TTBS) was prepared, containing 998 ml TBS and 2 ml Tween 20 (Acros Organics; Fisher Scientific). After western blotting, the cassettes were removed from the electrophoresis tank and unpacked. Membranes were removed from the gels, rinsed in TTBS, soaked in Ponceau S stain (diluted 1 in 10 with distilled water) and left to stand for 10 min. They were then rinsed with distilled water (until the protein bands became visible), labelled, wrapped in clingfilm and photographed with a Fujifilm LAS-3000 CCD camera. After imaging, the membranes were unwrapped from the clingfilm, and the positions of molecular weight markers on the protein ladders were marked on them with an antigen-antibody pen (for rabbit primary antibodies; Alpha Diagnostic, San Antonio, TX, USA), to enable subsequent visualisation with a CCD camera under chemiluminescent conditions. The remaining red stain was then washed off the membranes with TTBS and distilled water.

2.5.9 Immunodetection

The membrane was blocked overnight in 50 ml blocking solution (10 % weight/volume (w/v) milk powder (Marvel; Premier Foods, Spalding, Lincolnshire, UK) in TTBS) at 4 °C, then further blocked on a shaker at room temperature for 30 min. It was removed from the blocking solution, rinsed twice with TTBS, then incubated in 10 ml primary antibody solution (3 % w/v milk powder in TTBS, 1 in 10 000 UCP1 antibody) on a shaker at room temperature for 2 h. The antibody was raised in rabbit against sheep, and was developed in-house [Schermer et al, 1996]. The membrane was removed from the antibody solution, washed three times with TTBS on a shaker at room temperature for 10 min, then incubated in 10 ml secondary antibody solution (3 % w/v milk powder in TTBS, 1 in 2 000 antibody)

on a shaker at room temperature for 2 h. The secondary antibody was polyclonal swine anti-rabbit immunoglobulins with HRP (Dako, Ely, Cambridgeshire, UK). The membrane was removed from the antibody solution, washed four times with TTBS on a shaker at room temperature for 15 min, washed with TBS on a shaker for 30 min, then left to soak in TBS at room temperature for 45 min. It was then removed from the TBS and dried. Its surface was covered with 6 ml Immobilon Western Chemiluminescent HRP Substrate (Millipore, Watford, Hertfordshire, UK), comprising 3 ml peroxide solution and 3 ml luminol reagent, and it was incubated at room temperature for 5 min. It was then drained, wrapped in a new piece of clingfilm and imaged under chemiluminescence with a Fujifilm LAS-3000 CCD camera for 10 min. The protein band densities were analysed using Aida Advanced Image Data Analyzer software version 4 (Raytest, Straubenhardt, Germany).

2.6 Histology

2.6.1 Introduction

Histology is the anatomical study of the microscopic structure of organic tissues and cells. Basically it involves the preparation of very thin slices of tissue mounted on slides, which are then stained with dyes and/or incubated with labelled antibodies to identify different extracellular and intracellular structures, features and components. Samples of 7 day-old sternal adipose tissue from Study A that had been preserved in formal saline (Section 4.3.1.8, p 152) were processed, sectioned, stained and imaged, with a view to establishing if there was any difference in tissue morphology between the feeding groups. Immunohistochemistry (IHC) was also performed on a subset of these samples with a view to confirming the intracellular localisation of UCP1 protein detected by western blotting. No preserved samples were received from Denmark for Study B, and there was insufficient tissue to perform histology on the frozen samples.

2.6.2 Basic principles of histology

2.6.2.1 Tissue preservation and processing

As fresh tissue is fragile and easily damaged, it cannot be cut to the required thickness (around 5 μm) without first being preserved and supported. It is preserved or 'fixed' so that its cellular structure is maintained, and so that destruction or degradation of the tissue and its components and molecules by enzymes and microorganisms is prevented or minimised. It is supported so that it can be cut into thin sections without damaging it or compromising its morphology. These objectives can be achieved either by freezing the

tissue and cutting it into sections while still frozen, or by chemically fixing it and then embedding it in a liquid which subsequently solidifies. Common chemical fixatives are 10 % formalin[30] and formal saline (9 % w/v sodium chloride in 10 % formalin), and a common embedding medium is paraffin wax, which is liquid at around 60 °C, but sets hard at around 20 °C [Grizzle *et al*, 2008; Rolls, 2011].

A tissue needs to be 'processed' to transform it from its fixed state to its embedded state. A piece of preserved tissue, about 3–4 mm thick, is cut off and placed in a plastic perforated cassette. Processing then proceeds in four stages:

1. dehydration;

2. clearing;

3. infiltration;

4. embedding.

Dehydration is required because paraffin wax is hydrophobic and immiscible in water. Water (and fixative) is removed from the tissue by immersing it in a series of increasing concentrations of a dehydrating agent, such as ethanol or industrial methylated spirit (IMS). Clearing is the removal of the dehydrating agent with a 'clearing reagent' which is miscible in both the dehydrating agent and paraffin wax. Common clearing reagents include xylene, toluene and chloroform. Infiltration is the immersion of the tissue in liquid wax. Embedding is the process of mounting the infiltrated tissue into a solid wax block.

Tissue processing can be performed manually, but it is more common nowadays to use an automated tissue processor for the dehydration, clearing and infiltration stages. The most common type of automated tissue processor, and the type used in the study, is a 'fluid transfer' processor, where tissues are retained in one chamber for the duration of processing, and fluids (dehydration agent, clearing reagent and wax) are pumped in and out of the chamber as required. Such processors may also have facilities to fix the tissue before processing, and to regulate temperature and pressure to optimise processing and reduce its time.

Tissue is removed from the tissue processor after the infiltration stage, and the final embedding stage is often carried out at an embedding centre consisting of three modules:

- a heated storage console for moulds and cassettes;

- a wax dispensing console;

- a cold plate.

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[30] The liquid formalin (100 %) is a 37 % w/v solution of the gas formaldehyde.

The tissue is removed from its cassette, placed in a suitably-sized metal or plastic mould, and orientated. The mould is filled with liquid wax, the cassette is placed on top of the mould, and the mould-cassette combination is placed on the cold plate. When the wax has solidified, the mould is removed from the cassette, leaving the tissue embedded in a wax block, on top of the cassette, ready for cutting with a microtome. Tissue embedded in a wax block is stable, and can be retained almost indefinitely. Sections cut from the block are floated in a heated water bath to be flattened out by surface tension, transferred to a glass slide, and dried on a hot plate and/or in an oven [Rolls, 2011; Spencer & Bancroft, 2008a; b].

2.6.2.2 Staining

Different stains are used to visualise different cellular structures and features. A stain is either a dye or a reagent used to produce a dye. Some dyes need to form a complex with a 'mordant', usually a metallic salt, in order to bind to tissue. Haematoxylin and eosin (H&E) is the most widely used histological stain, and is the one used in this study. Haematoxylin is not a dye, but is oxidised (usually with sodium iodate or mercuric oxide) to produce the dye haematein. Haematein is anionic and needs a mordant, usually an aluminium-based salt such as potassium aluminium sulfate. The metallic ion of the mordant confers a net positive charge on the dye-mordant complex, enabling it to bind to negatively-charged sites such as nuclear chromatin. The oxidising agent and mordant are incorporated into commercial haematoxylin staining solutions. Haematein stains nuclei blue-black, while eosin stains the cytoplasm and connective tissue fibres in different shades of orange, pink and red. In contrast to haematein, eosins are xanthene dyes and do not need an oxidising agent or mordant. Differentiation of eosins is achieved by washing with tap water, and by subsequent dehydration.

The procedure for staining involves dewaxing the tissue with a clearing reagent and rehydrating it with decreasing concentrations of alcohol and ultimately water. It is then stained with a haematoxylin solution. It can either be 'progressively' stained, whereby the stain is added for just sufficient time to colour the nuclei without staining the background, or 'regressively', where it is overstained and then 'differentiated' using an acid alcohol solution. Nuclei are initially stained red, but washing the tissue with a weak alkaline solution such as Scott's tap water achieves the required blue-black colour, a process termed 'blueing'. The tissue is then washed, stained with eosin, washed again, dehydrated and cleared. Finally, a transparent cover slip is fixed over the tissue with an adhesive, and the slide is left to dry. The stained sample can be visualised and/or photographed through a microscope [Gamble, 2008; Horobin, 2008].

2.6.2.3 Immunohistochemistry (IHC)

IHC is a technique for detecting and localising components of a histological tissue section by antigen-antibody interactions. The process is analogous to the immunodetection stage of western blotting (Section 2.5.9, p 121; Figure 2.18, p 117) where a labelled antibody is used to identify a specific protein. As in western blotting, the detection method can be direct, where a label is linked to a primary antibody, or indirect, where it is linked to a secondary antibody. Several molecules of secondary antibody can also be linked by a polymer chain to multiple label molecules, increasing the assay sensitivity and allowing secondary antibodies against different species (eg rabbit and mouse) to be used in the same reagent. As in western blotting, labels can be radioactive, fluorescent or enzymatic, though radioactive labelling is rare, and it is difficult to show the morphology of a cell with fluorescence (which is visualised through a fluorescence microscope). Enzymes are therefore the most commonly used labels with IHC, but they are usually used with a chromogen (a substance that can be converted into a dye or pigment) rather than a chemiluminescent substrate. Use of a chromogen enables the end-product to be visualised though a light microscope and, if the tissue is counterstained (eg with haematoxylin), allows the specific target of IHC and the surrounding cellular morphology to be viewed simultaneously. A common label used in IHC is the enzyme HRP, often in combination with the chromogen 3,3'-diaminobenzidine (DAB) tetrahydrochloride, which produces a stable, insoluble, dark brown pigment. If enzymes similar to those used as the label are present in the tissue (eg endogenous peroxidases in the case of an HRP label), they would also react with the substrate, so 'blocking' is required to prevent non-specific staining [Jackson & Blythe, 2008].

A problem in IHC which does not occur in western blotting arises from the process of fixing tissues. When formalin-based fixatives are used, cross-linkages are formed with certain structural proteins which mask antibody-binding sites (ie epitopes). These linkages need to be broken down to unmask the epitopes. Unmasking is achieved either by proteolytic enzyme digestion or by heat-induced epitope retrieval, which involves heating the tissue in the presence of a buffer, such as citrate or EDTA [Jackson & Blythe, 2008].

Tissues are fixed and processed, and slides are prepared, as described in Section 2.6.2.1, p 122. The IHC process involves dewaxing and rehydration of the tissue, epitope retrieval, blocking of endogenous enzymes, incubation with the primary antibody, incubation with the secondary antibody, incubation with the substrate, application of the counterstain (if applicable), dehydration, clearing, and application of a cover slip. The procedure can be carried out manually, but most of the steps can now be automated.

2.6.3 Tissue processing

As described in Section 4.3.1.8, p 152, samples of Study A adipose tissue were preserved in formal saline for histology. A small piece of preserved tissue was cut from each sample and placed in a 30 mm × 25 mm × 5 mm Histosette II tissue processing/embedding cassette (Simport Scientific, Beloeil, QC, Canada). The cassettes were immersed in 70 % IMS pending processing, then processed overnight in a Shandon Excelsior advanced tissue processor (Thermo Shandon Ltd, Runcorn, Cheshire, UK). The processing programme comprised six cycles of IMS at 20 °C for 1 h, three cycles of xylene (Fisher Scientific) at 20 °C for 1 h, and three cycles of wax (Tissue-Tek Embedding Wax; Sakura Finetek, Alphen aan den Rijn, Netherlands) at 62 °C for 1 h 20 min. The IMS concentrations were graduated, with the first three cycles being 75 %, 90 % and 95 % respectively, and the remaining three cycles being 100 %. After processing, each sample was embedded in a paraffin wax block using a Tissue-Tek III Embedding Center (Sakura Finetek). For each sample, 5 × 5 μm sections were sliced from the block using a sledge microtome (AS200; Anglia Scientific, Cottenham, Cambridge-shire, UK), rinsed in 70 % IMS, then floated in a water bath at 45 °C. They were then transferred onto SuperFrost Plus glass microscope slides (Menzel-Gläser; Gerhard Menzel, Braunschweig, Germany), dried on a heat rack for 30 min, then further dried in an oven at 37 °C for at least 24 h.

2.6.4 H&E staining

Tissue slides were placed in a slide rack, and tissues were dewaxed and rehydrated by successive 3 min immersions in xylene (twice), 100 % IMS (twice), 70 % IMS and distilled water. They were then stained for 5 min with Harris's haematoxylin, rinsed in running tap water for 5 min to remove excess stain, differentiated by dipping briefly in acid alcohol (1 % concentrated hydrochloric acid in 70 % IMS), blued by immersion in Scott's tap water (0.2 % w/v potassium bicarbonate, 2 % w/v magnesium sulfate) for 1½ min, and rinsed once more in running tap water for 5 min. They were subsequently stained with 0.1 % w/v eosin yellowish (BDH, Gurr Certistain; VWR) for 3 min, rinsed in running tap water for 1½ min, dehydrated by two successive immersions in 100 % IMS for 2 min, and cleared by two successive immersions in xylene, for 2 and 3 min respectively. Glass cover slips (Fisherbrand; Fisher Scientific) were then affixed to slides with DPX mounting medium (Fisher Scientific), such that they covered the stained tissue, and slides were left to dry overnight.

Stained slides were visualised through a Leica DMRB microscope (Leica Microsystems, Milton Keynes, UK) and photographed with a Retiga-2000R CCD digital camera (QImaging, Surrey, BC, Canada) at 20X magnification. Images were saved in 'BigTIFF' format, then

processed with Image-Pro Premier software (Media Cybernetics, Rockville, MD, USA) to produce images in TIFF format. For each sample, a representative 2 mm × 2 mm region of tissue was selected and an image produced. Images of 1 mm × 1 mm and 0.5 mm × 0.5 mm regions respectively were then produced by progressively 'drilling down' from the initial 2 mm × 2 mm image. By comparing samples of the same size (0.5 mm × 0.5 mm) and at the same magnification, tissues were analysed qualitatively in terms of cell number, size and morphology. Lack of time precluded the use of more quantitative techniques.

2.6.5 Immunohistochemistry

Slides of tissue sections were prepared as described in Section 2.6.3, p 126. Two slides were selected for each sample, one to be used with the UCP1 primary antibody, and one to be used as a negative control. Tissues were processed with a Vision BioSystems Bond-maX automated IHC slide processor (Leica Microsystems) controlled by Leica Bond software version 4.0, using a Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK). The kit contained Peroxide Block (3–4 % w/v hydrogen peroxide), Post Primary (rabbit anti-mouse IgG, < 10 µg ml^{-1}, in 10 % volume/volume (v/v) animal serum in TBS/ 0.09 % ProClin 950), Polymer (anti-rabbit poly-HRP-IgG, < 25 µg ml^{-1}, containing 10 % v/v animal serum in TBS/0.09 % ProClin 950), DAB Part 1 (66 mM DAB tetrahydrochloride in a stabiliser solution), DAB Part 2 (≤ 0.1 % v/v hydrogen peroxide in a stabiliser solution), and Hematoxylin (< 0.1 % haematoxylin). Tissues were first dewaxed by 3 applications of Bond Dewax Solution (organic solvent containing > 98 % alkanes): an incubation for 30 s at 72 °C; a rinse at 72 °C; and a rinse at ambient temperature. This was followed by 3 rinses with 100 % IMS, and 3 applications of Bond Wash Solution (supplied as 10X concentrate containing TBS, surfactant and 3.5 % ProClin 950, pH 7.6): 2 rinses; and an incubation for 5 min. Epitopes were retrieved by 4 applications of Bond Epitope Retrieval Solution 1 (a citrate-based buffer, pH 6.0): 2 rinses at ambient temperature; an incubation for 20 min at 100 °C; and an incubation for 12 min at ambient temperature. This was followed by 4 applications of Bond Wash Solution: 3 rinses at 35 °C; and a 3 min incubation at ambient temperature. Blocking of endogenous peroxidases was effected by incubation with Peroxide Block for 5 min. This was followed by 3 rinses with Bond Wash Solution. Tissues other than the negative controls were then incubated with 150 µl primary antibody against UCP1, diluted 1 in 750 (determined by previous assay to produce the most specific staining) in Bond Primary Antibody Diluent (containing TBS, surfactant, protein stabiliser and 0.35 % ProClin 950), for 15 min. This was followed by 3 rinses with Bond Wash Solution. Tissues were then

incubated in Post Primary for 8 min,[31] followed by 3 incubations for 2 min with Bond Wash Solution. They were then incubated with 150 µl secondary antibody, Polymer, for 8 min. This was followed by 2 incubations with Bond Wash Solution for 2 min and a rinse with distilled water. Tissues were then rinsed with 150 µl substrate, Mixed DAB Refine (DAB Part 1 and DAB Part B in a ratio of 1:25 by volume), and incubated in it for 10 min. This was followed by 3 rinses with distilled water. They were then incubated with 150 µl counter-stain, Hematoxylin, for 5 min. This was followed by a rinse with distilled water, a rinse with Bond Wash Solution, and a final rinse with distilled water. Slides were removed from the Bond-maX machine, dehydrated by 2 successive immersions in 100 % IMS for 2 min, and cleared by 2 successive immersions in xylene, for 2 and 3 min respectively. Glass cover slips were then affixed to the slides, and slides were visualised, photographed and imaged, as detailed in Section 2.6.4, p 126.

2.7 Statistics

2.7.1 Analysis

All statistical analyses were performed with the free statistical programming language and environment R [R Core Team, 2014]. Shapiro-Wilk and Anderson-Darling tests were used to determine whether data were normally distributed, with a normal distribution being assumed only if both tests were passed, and where visual examination of frequency histograms and normal Q–Q plots was consistent with the test results. If tests on the original data failed to show a normal distribution, the process was repeated with log-transformed data. In the event, no data passed the normal distribution tests, and all statistical tests were therefore non-parametric. A Mann-Whitney U-test was used to compare two groups, and a Kruskal-Wallis test was used to compare more than two groups. In the latter case, a *post hoc* Dunn's test was used where appropriate. For tests of relationships, a Spearman's correlation was used.

Results of the Mann-Whitney and Dunn's tests were deemed significant if p was less than 0.05. However, in view of the small sample sizes and low statistical power of some tests, it was considered useful additional information to also report the value of p if it fell between 0.05 and 0.1. The phrase 'weak evidence' is used in the text when describing such results. To be consistent with this approach, Kruskal-Wallis tests were evaluated using a $p < 0.1$ criterion, but it is noted in the text where $0.05 \leq p < 0.1$.

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[31] This step has no functional significance for this study, as Post Primary is an IgC linker reagent which localises mouse antibodies.

No adjustment was made for multiple comparisons (eg Bonferroni), as it was felt that the advantage of such adjustments, being a reduction in the number of false positives, was outweighed by the disadvantages. The latter include an increase in the number of false negatives, subjectivity in choosing which comparisons to adjust for, and various technical reasons [Perneger, 1998]. The approach has therefore been to disclose differences between sample groups where p is less than 0.1, to report the actual value of p, and to describe the various comparisons that were made.

2.7.2 Graphical presentation

Data are presented as the mean of each sample group, with error bars representing the standard error of the mean (SEM). Where statistical comparisons have been made and p is less than 0.05, a solid horizontal line has been drawn above the relevant columns in the bar charts and scatter plots shown in the Results sections. Where p falls between 0.05 and 0.1, a dashed horizontal line is used instead. A lower case letter above each line is a key to the p-value, which is disclosed in the figure caption.

2.7.3 Power calculations

Two errors can occur in statistical hypothesis testing: rejection of the null hypothesis (H_0) when it is in fact true (a Type I error or false positive); and acceptance of H_0 when it is in fact false (a Type II error or false negative). The maximum probability of a Type I error (α) is the significance level set for a specific statistical test. This is conventionally set at 0.05 in the biological and biomedical sciences, meaning that there is a 5 % probability that a true H_0 will be rejected (ie 5 % probability of a false positive). The probability of a Type II error is denoted by the Greek letter β.

The 'power' of a statistical test is the probability that H_0 will be rejected when it is false (which is equivalent to $1 - \beta$). Tests that lack statistical power are of limited use, because they cannot reliably distinguish between H_0 and the alternative hypothesis of interest (H_1). The power is a function of the chosen α, the sample size (n) and the effect size (ρ), and any three of these variables can be used to calculate the fourth. In an *a priori* calculation, which is performed before an experiment, the power, significance level and desired effect size are specified in order to determine the appropriate sample size. Although any power figure can be specified, a figure of 0.8 (80 %) is conventionally used, meaning that there is no more than a 20 % (1 – 0.8) probability of making a Type II error. This figure was proposed by Cohen [1988], who reasoned that a 4:1 ratio was a good balance between β and α risk (ie that it was more important to avoid false positives than false negatives). Alternatively, a

post hoc calculation can be performed after a hypothesis test to retrospectively assess its power and/or to determine the sample size needed to achieve the desired power.

No *a priori* calculations were performed in either study, but, where appropriate, *post hoc* power calculations were carried out using G*Power software v 3.1.9.2 [Faul *et al*, 2007]. In calculating ideal sample sizes, a desired power of 80 % was specified. There are limitations in using power calculations for non-parametric tests, because while their validity does not depend on a specific distribution, a distribution must nevertheless be specified in order to estimate numerical values for the power [HHU, 2014]. The distribution options specified in the software for a Mann-Whitney U-test are 'Normal', 'Laplace', 'Logistic' or 'min ARE' (which is minimal asymptotic relative efficiency). The 'min ARE' option was selected in all cases, as it gives the most conservative result (lowest power or largest sample size).

3 The ontogeny of gene expression in ovine sternal adipose tissue in early postnatal life

3.1 Introduction

The sternal adipose tissue depot in sheep is the equivalent of the supraclavicular depot in humans, which is the primary site of functional BAT in adults [Nedergaard *et al*, 2007]. As far as can be ascertained, it has not been characterised previously, so the principal aim of the study was to examine changes in gene expression over the first 28 days of postnatal life, and to compare them to those that have been reported for perirenal adipose tissue, the principal depot in newborn sheep [Symonds *et al*, 2012a]. The ontogeny of tissue weights for the sternal and perirenal adipose tissue depots was also examined, with a view to determining whether there is any correlation between changes in gene expression and changes in fat mass. A further aim of the study was to ascertain whether UCP1-expressing adipocytes in ovine sternal adipose tissue were of classical brown or brite/beige lineage.

3.2 Hypotheses

In sheep, as in other precocial mammals, BAT rapidly atrophies after birth and is replaced by WAT, reflecting a diminishing requirement for NST as the animal grows [Cannon & Nedergaard, 2004; Casteilla *et al*, 1989; Gemmell *et al*, 1972]. It was therefore hypothesised that there would a transition from a brown or mixed phenotype to a white phenotype over the first month after birth, with a corresponding reduction in the expression of thermogenic and BAT or brite/beige marker genes. In contrast, it was hypothesised that the increase in fat mass with growth would be a function of both hypertrophy and hyperplasia, so it was expected that the expression of genes involved in adipogenesis and metabolism would increase with age.

The hypotheses for this chapter, therefore, were that in the sternal adipose tissue of newborn lambs:

- the ontogeny of gene expression over the first month after birth would be similar to that reported for perirenal adipose tissue;

- expression of thermogenic and BAT or brite/beige adipose tissue marker genes would reduce over the first month after birth;

- expression of adipogenic and metabolic genes would increase over the first month after birth;

- UCP1-expressing adipocytes would be brite/beige rather than brown.

3.3 Materials and methods

3.3.1 Experimental procedures

This study was complementary to the nutritional study (Study A) which is presented in Chapter 4, so the materials and methods are a subset of those used in that chapter. The latter study looked at the effect of maternal fat supplements on the sternal adipose tissue of suckling lambs at 7 and 28 days of age. This study only uses control animals, to confound the effect of the dietary supplements, but also includes an additional 1 day-old group comprising five animals (one male, four females) sampled at 1–3 days, all from triplets. The experimental protocol for this study is shown in Figure 3.1, p 133. The overall protocol and husbandry procedures are detailed in Section 4.3.1, p 149, and the laboratory procedures for gene expression are described in Section 2.4, p 70.

3.3.2 Statistics

The overall methodology for statistical analysis and presentation of data is described in Section 2.7, p 128. Where Kruskal-Wallis tests were used to compare all three age groups (1, 7 and 28 days of age), the p-value is displayed at the top right-hand side of each sub-figure. If p was less than 0.1, a *post hoc* Dunn's test was then carried out between each pair of age groups, but no adjustments were made to p-values for multiple comparisons. There was only one male lamb in the 1 day-old group, so no statistical comparisons could be carried out with this group when presenting the results by sex.
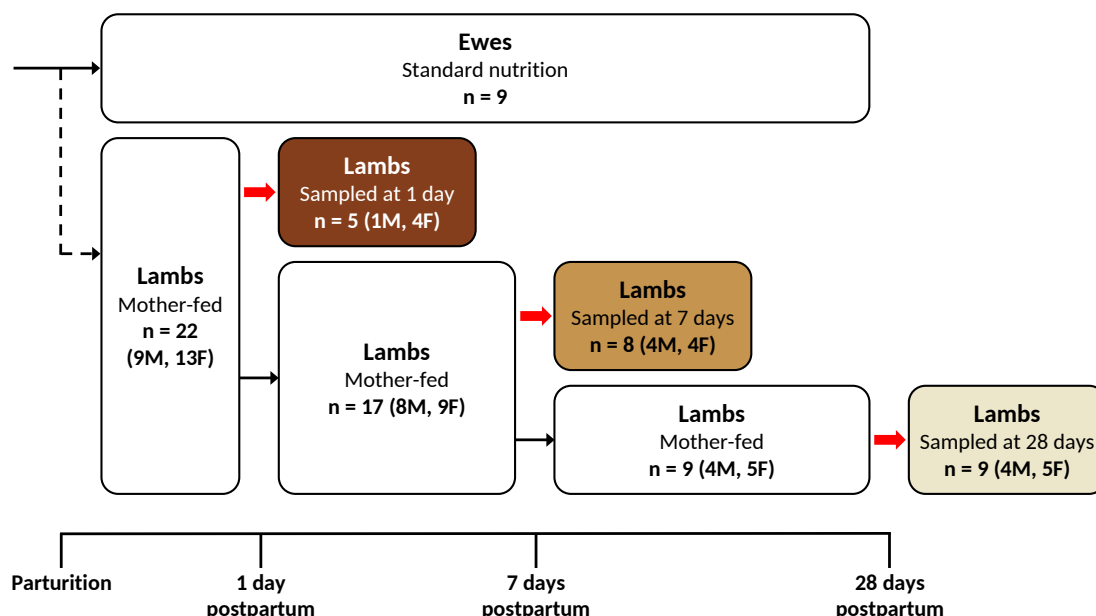
Figure 3.1 Experimental protocol. The full protocol (excluding lambs sampled at 1 day of age) is shown in Figure 4.1, p 150. Pregnant ewes were fed a standard diet until parturition, and were then allowed to lamb normally at term (21 weeks). After parturition, the ewes remained on a standard diet, and their lambs were mother-fed until they were humanely euthanased and sampled. Five lambs from triplets were sampled at around 1 (+ 2) day of age. One lamb from each twin pair or triplet was sampled at around 7 (± 1) days of age, and the remaining lamb at around 28 (± 3) days of age. A singleton male lamb (sibling died) was sampled at 28 days of age. M is male and F is female. The colour coding is intended to facilitate interpretation of the results figures in this chapter.

In determining effect sizes for statistical power calculations on tests of tissue weights and gene expression, the absolute percentage differences between the means of the two age groups compared, and the coefficients of variation for each group, were averaged across all tissues or genes. The G*Power software does not have an option for a Dunn's test, so the option for a Mann-Whitney U-test was selected instead.

3.4 Results

3.4.1 Tissue weights

The tissue weight profiles for both sternal and perirenal adipose depots were similar for males and females, so results are shown below for the combined sexes (Figure 3.2, p 134). By way of further information, results for the individual sexes are also shown in Appendix B, Figure B-1, p 283. The overall rate of growth of the two tissues from 1 to 28 days of age was not dissimilar, with the increases being some 8-fold and 9-fold respectively for sternal and perirenal adipose tissue in absolute terms, and around 2-fold and 2½-fold relative to body weight. However, whereas the relative weight of perirenal adipose tissue increased more rapidly from 1 to 7 days of age than thereafter, the relative weight of sternal adipose
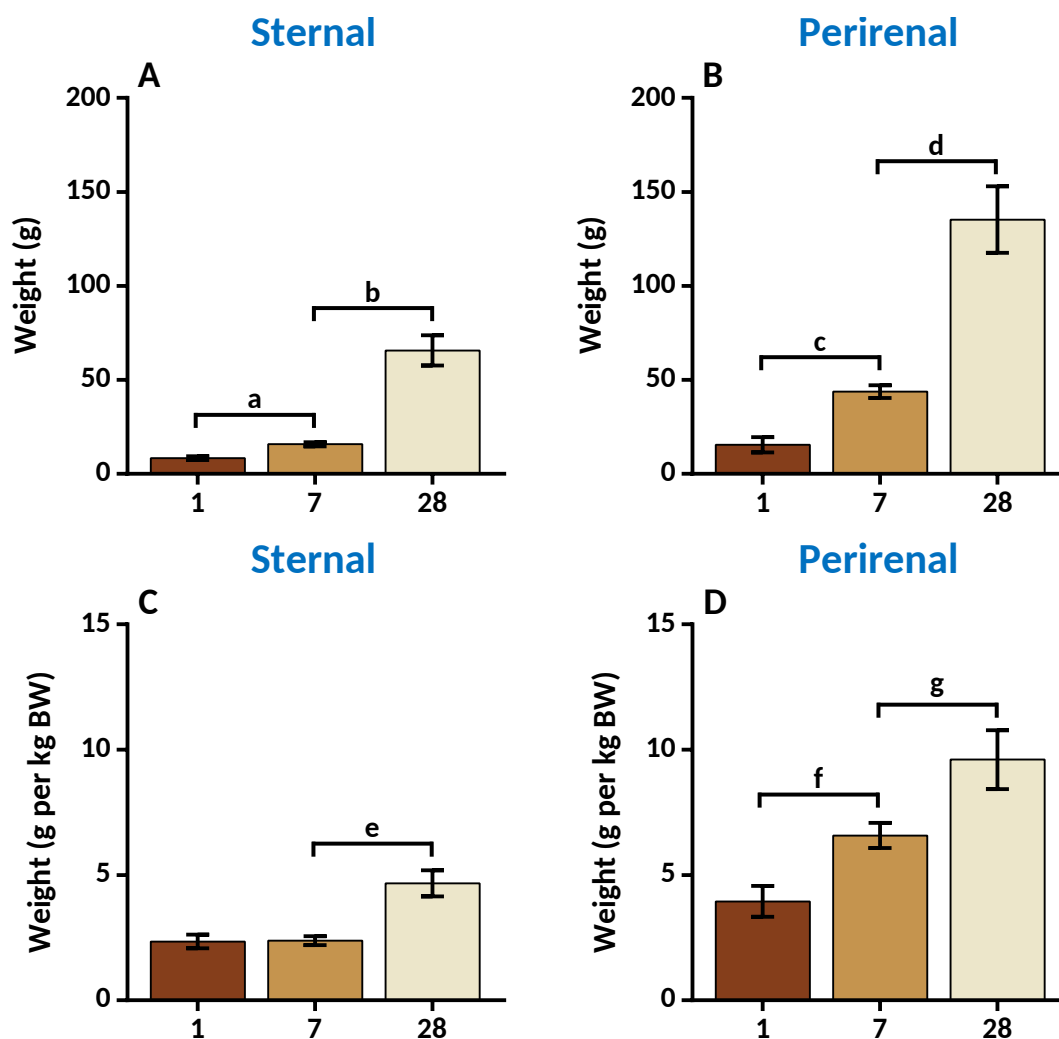
Figure 3.2 Sternal and perirenal adipose tissue weights by age. Values shown are mean tissue weights ± SEM of lambs sampled at 1 (+ 2; n = 4/5; 1M, 3/4F), 7 (± 1; n = 8; 4M, 4F) and 28 (± 3; n = 9; 4M, 5F) days of age, both in absolute terms (**A**, **B**) and relative to body weight (BW) (**C**, **D**). M is male, F is female. The 1–7 and 7–28 groups were compared by Mann-Whitney U-tests (a: $p = 0.0016$; b: $p = 0.0001$; c: $p = 0.0016$; d: $p = 0.0001$; e: $p = 0.0010$; f: $p = 0.0162$; g: $p = 0.0206$; $p > 0.1$ in the other case).

tissue was unchanged from 1 to 7 days of age, with the whole of the increase occurring between 7 and 28 days of age.

3.4.2 Gene expression

The gene expression profile for male and female lambs was similar in most cases, so these results are also shown for the combined sexes, and are presented in terms of normalised DNA copy number: Figure 3.3, p 135 (adipogenic genes), Figure 3.4, p 136 (metabolic genes), Figure 3.5, p 137 (thermogenic genes), and Figure 3.6, p 138 (developmental genes). By way of further information, the results by sex are shown in Appendix B, Figure B-2, p 284 (adipogenic genes), Figure B-3, p 285 (metabolic genes), Figure B-4, p 286 (thermogenic genes), and Figure B-5, p 287 (developmental genes).

The most highly expressed gene at all three time points was FABP4 (around 1.4, 1.7 and 2.1 million DNA copies respectively). Expression of UCP1 was the second highest at 1 day of age and the third highest at 7 days of age (around 150 000 and 130 000 copies respectively), but had reduced to virtually zero (179 copies) by 28 days of age. Adiponectin had the third highest expression at 1 day of age and the second highest expression at 7 and 28 days of age (around 133 000, 366 000 and 528 000 copies respectively). PPARγ was the next most highly expressed gene (around 22 000, 35 000 and 46 000 copies respectively for 1, 7 and 28 days of age). The only other genes that expressed more than 10 000 DNA copies at any of the three ages were PGC1α (1 and 7 days of age only), NR3C1 and C/EBPα.
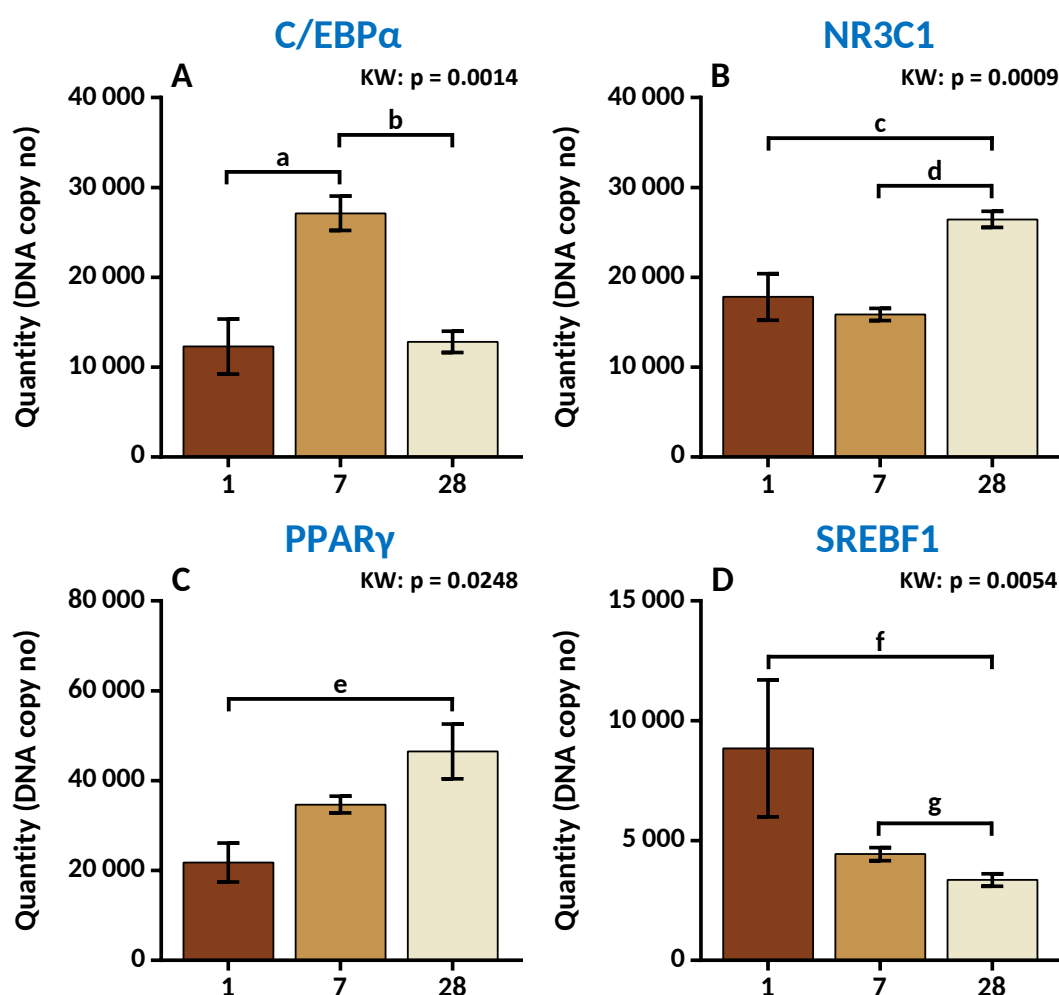
Figure 3.3 Expression of adipogenic genes in sternal adipose tissue by age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; n = 5; 1M, 4F), 7 (± 1; n = 8; 4M, 4F) and 28 (± 3; n = 9; 4M, 5F) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. Age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (a: p = 0.0032; b: p = 0.0013; c: p = 0.0105; d: p = 0.0004; e: p = 0.0067; f: p = 0.0019; g: p = 0.0373; p > 0.1 in all other cases). No adjustments have been made to p-values for multiple comparisons.
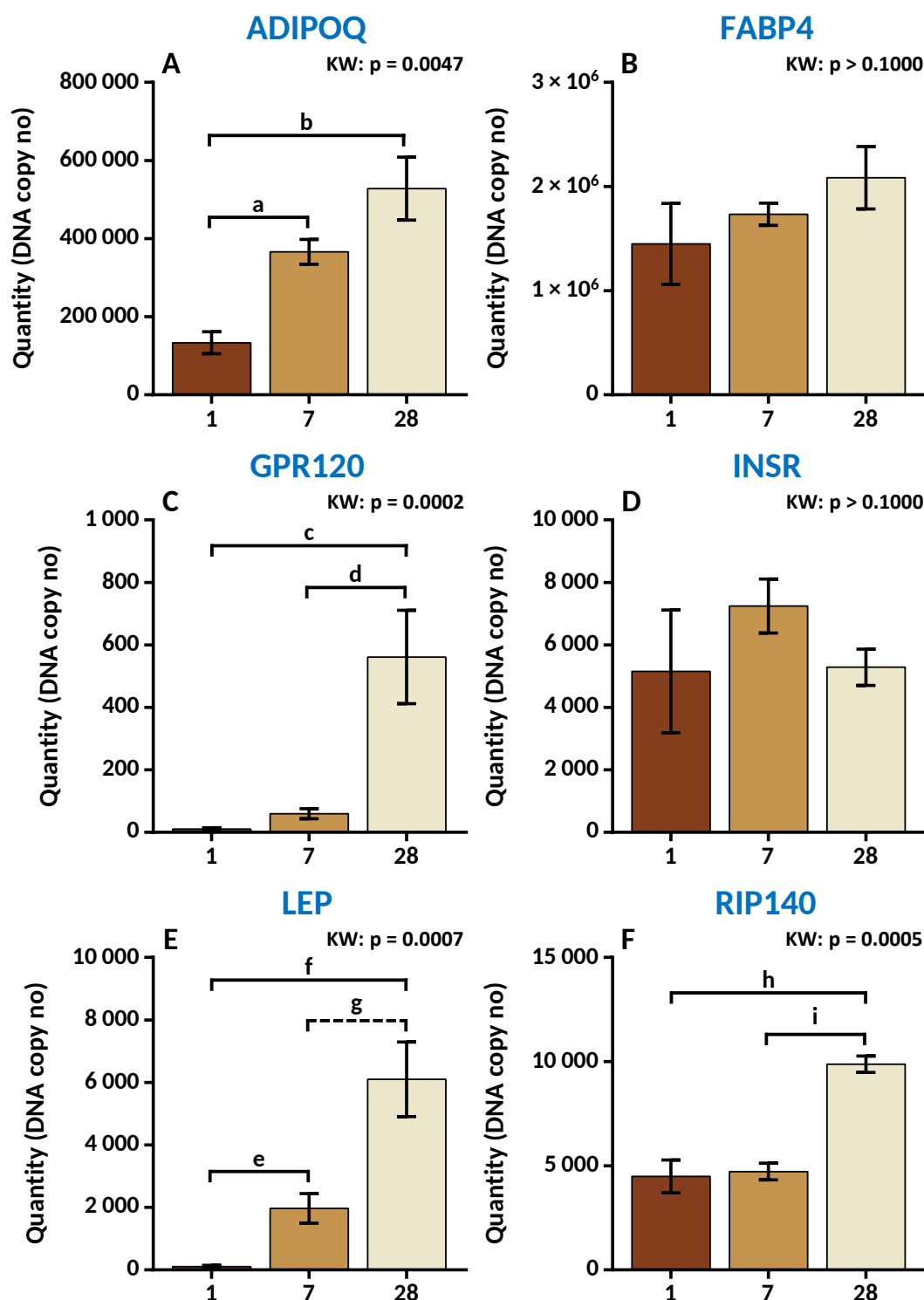
Figure 3.4 Expression of metabolic genes in sternal adipose tissue by age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; n = 5; 1M, 4F), 7 (± 1; n = 8; 4M, 4F) and 28 (± 3; n = 9; 4M, 5F) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. Age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (a: p = 0.0345; b: p = 0.0011; c: p = 0.0001; d: p = 0.0070; e: p = 0.0334; f: p = 0.0001; g: p = 0.0614; h: p = 0.0020; i: p = 0.0006; p > 0.1 in all other cases). No adjustments have been made to p-values for multiple comparisons.
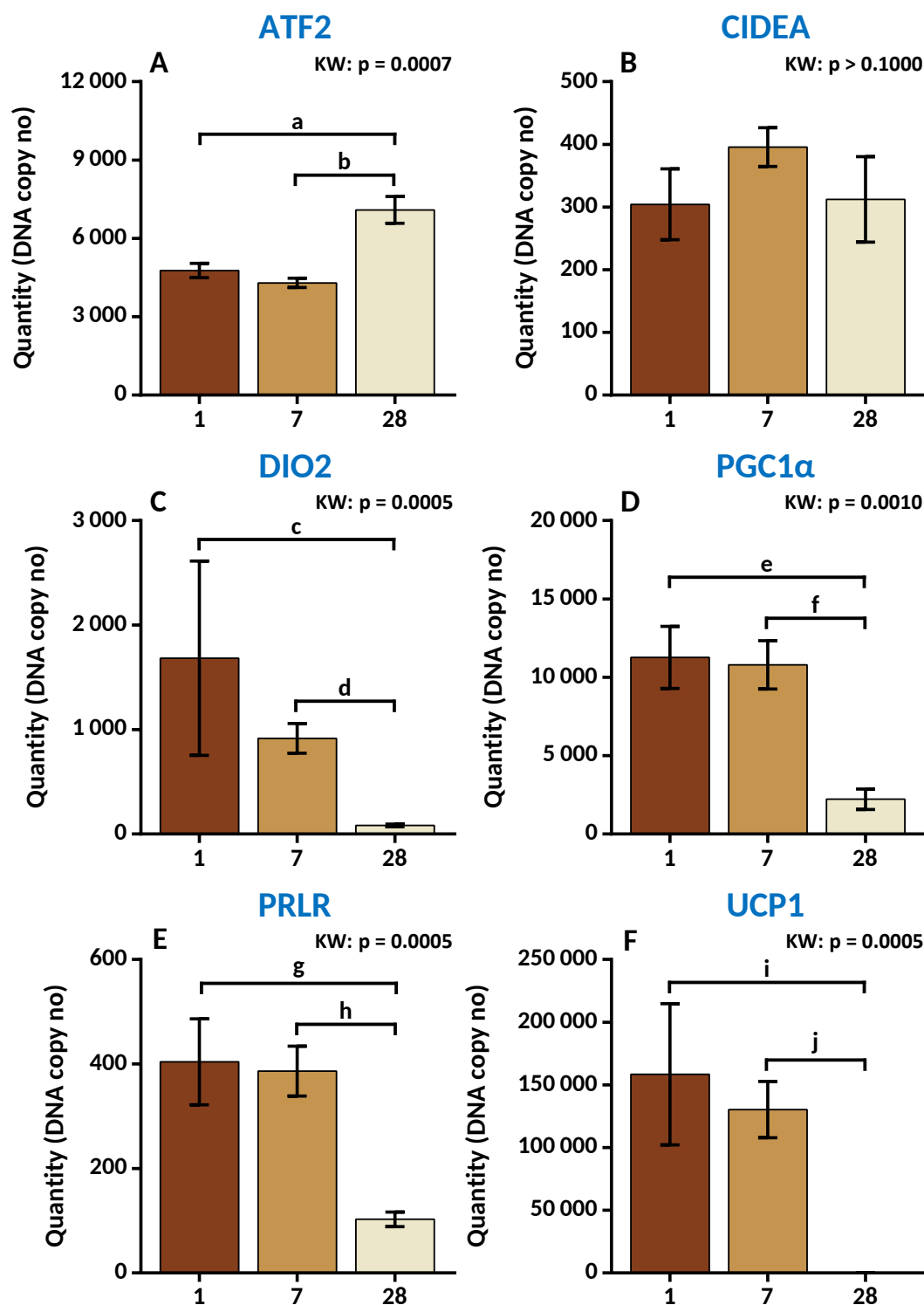
Figure 3.5 Expression of thermogenic genes in sternal adipose tissue by age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; n = 5; 1M, 4F), 7 (± 1; n = 8; 4M, 4F) and 28 (± 3; n = 9; 4M, 5F) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. Age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (a: p = 0.0194; b: p = 0.0002; c: p = 0.0011; d: p = 0.0009; e: p = 0.0032; f: p = 0.0011; g: p = 0.0016; h: p = 0.0007; i: p = 0.0011; j: p = 0.0009; p > 0.1 in all other cases). No adjustments have been made to p-values for multiple comparisons.
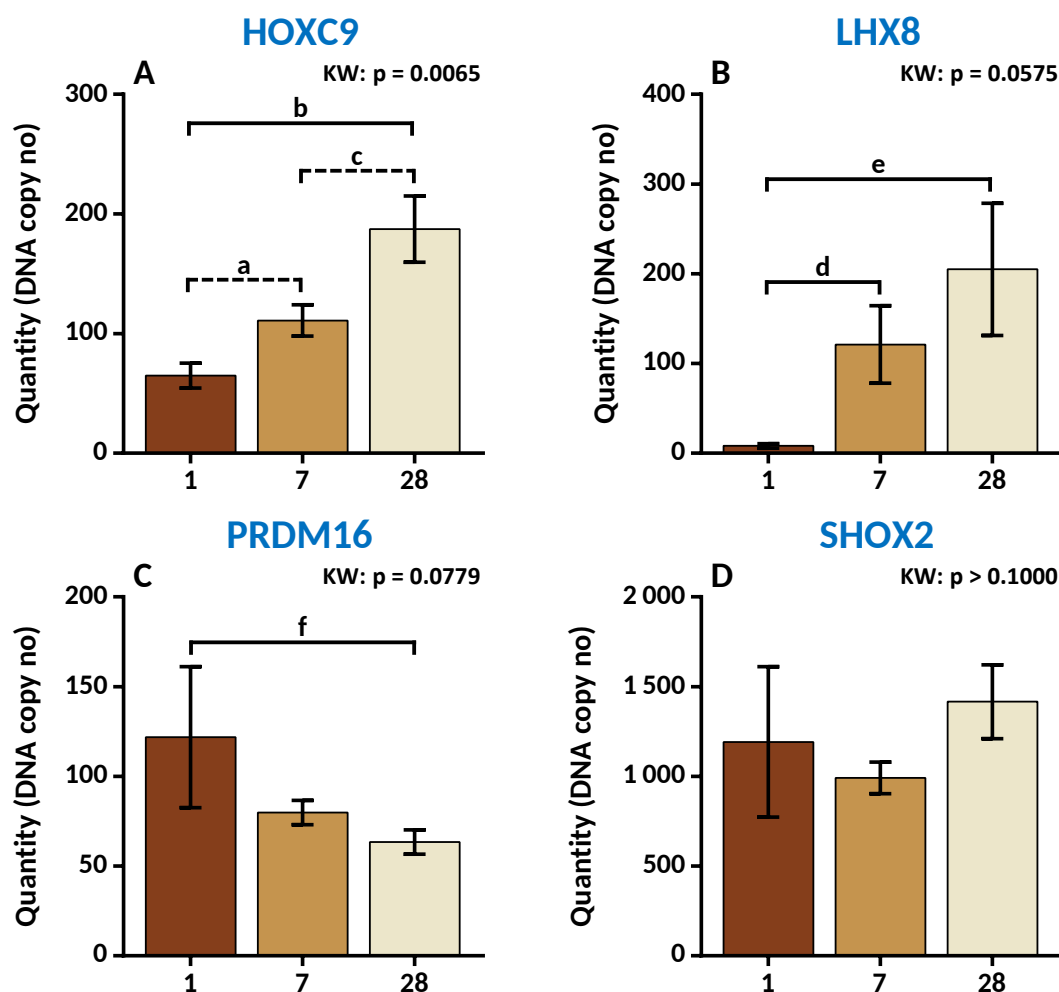
Figure 3.6 Expression of developmental genes in sternal adipose tissue by age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; n = 5; 1M, 4F), 7 (± 1; n = 8; 4M, 4F) and 28 (± 3; n = 9; 4M, 5F) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. Age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (a: p = 0.0967; b: p = 0.0016; c: p = 0.0961; d: p = 0.0340; e: p = 0.0285; f: p = 0.0313; p > 0.1 in all other cases). No adjustments have been made to p-values for multiple comparisons.

In general, genes classified as adipogenic or metabolic showed an increase in expression with age, whereas those classified as thermogenic showed a reduction. Exceptions to the general trend in the adipogenic and metabolic categories were C/EBPα, whose expression peaked at 7 days, SREBF1, whose expression declined with age, and FABP4 and INSR, whose expression did not significantly change with age. Exceptions in the thermogenic category were ATF2, whose expression increased with age, and CIDEA, whose expression did not significantly change with age. There was no common pattern of expression with developmental genes. Although in some cases the absolute level of expression of certain genes was higher or lower in males than in females, the change from 1 to 28 days, as noted above, was similar for both sexes. However, while the profiles were similar, the smaller sample sizes resulted in higher p-values, so significance was either reduced or lost.

3.4.3 Statistical power

The average effect sizes for differences in relative tissue weights between the three age groups was sufficiently large (ranging from around 2.1 to 3.8) that power was calculated to be above the conventionally accepted level of 80 % for all three tests (1–7 days, 87 %; 1–28 days, 100 %; 7–28 days; 99 %). The average effect sizes for differences in gene expression were lower (ranging from around 1.6 to 1.8), but power was still close to the accepted level for two of the three comparisons (1–28 days, 79 %; 7–28 days; 81 %). Power for the 1–7 days comparison was only around 62 %, and calculations indicates that the sample size would need to be increased from 5 and 8 (ie a total of 13) to 9 in each group (ie a total of 18) to achieve a power of 80 % with the same effect size.

3.4.4 Summary of results

A summary of the results for this chapter is shown in Table 3.1, p 140.

3.5 Discussion

3.5.1 Tissue weights

3.5.1.1 The increase in tissue weight is delayed in the sternal adipose depot of sheep compared to the perirenal depot

Although the overall rate of growth from 1 to 28 days of age was similar for sternal and perirenal adipose tissue, the latter increased faster initially, with the growth of the former effectively being delayed. This is most obvious when comparing tissue weights relative to body weight, where the relative tissue weight of the sternal depot was unchanged from 1 to 7 days of age, while that of perirenal adipose tissue had increased by some 70 %. It is not clear why there should be preferential deposition of fat in the perirenal depot in the first week of postnatal life, though UCP1 is still being expressed at high levels in that period, so it may relate to the need to generate heat close to a vital organ (ie the kidney) in the first few days after birth. There is no apparent correlation between tissue weight profiles and any gene expression profiles in the two tissues.

Table 3.1 Summary of results. Periods are from 1 to 7 days, 1 to 28 days, and 7 to 28 days. An up arrow (↑⇧) indicates that tissue weight or gene expression has increased over the period, a down arrow (↓) indicates that gene expression has decreased over the period, and a horizontal dash (–) indicates no change in tissue weight or gene expression over the period (p > 0.1). An outline arrow (⇧) indicates weak evidence of a change (ie $0.05 \leq p < 0.1$).

Item	Category	Tissue/gene	Period		
			1–7	1–28	7–28
Tissue weight	Absolute	Sternal	↑		↑
		Perirenal	↑		↑
	Relative	Sternal	–		↑
		Perirenal	↑		↑
Gene expression	Adipogenesis	C/EBPα	↑	–	↓
		NR3C1	–	↑	↑
		PPARγ	–	↑	–
		SREBF1	–	↓	↓
	Metabolism	ADIPOQ	↑	↑	–
		FABP4	–	–	–
		GPR120	–	↑	↑
		INSR	–	–	–
		LEP	↑	↑	⇧
		RIP140	–	↑	↑
	Thermogenesis	ATF2	–	↑	↑
		CIDEA	–	–	–
		DIO2	–	↓	↓
		PGC1α	–	↓	↓
		PRLR	–	↓	↓
		UCP1	–	↓	↓
	Development	HOXC9	⇧	↑	⇧
		LHX8	↑	↑	–
		PRDM16	–	↓	–
		SHOX2	–	–	–

3.5.2 Gene expression

3.5.2.1 The ontogeny of gene expression in ovine sternal adipose tissue in the first month of postnatal life is similar to that of perirenal adipose tissue

As indicated in Section 3.1, p 131, it was felt that it would be interesting to compare the changes in gene expression over the first month of postnatal life observed in ovine sternal adipose tissue to results previously reported by both our research group and others for perirenal adipose tissue. A total of 13 genes were common to both this study and one such study by our group [Pope et al, 2014]: the adipogenic genes NR3C1 (glucocorticoid receptor) and PPARγ; the metabolic genes ADIPOQ (adiponectin), FABP4, LEP (leptin) and RIP140; the

thermogenic genes CIDEA, DIO2, PGC1α, PRLR and UCP1; and the developmental genes HOXC9 and PRDM16. The cited study tested expression at 30, rather than 28, days of age, but that minor difference is ignored for the purposes of the following discussion. It also examined two prenatal time points, and used a Kruskal-Wallis test to compare all five groups (two prenatal and three postnatal), so potentially some significant differences may have been lost that would have been apparent if the test had been restricted to postnatal groups. Notwithstanding that, the expression profiles of leptin and RIP140 were similar for both studies, with a general and significant upward trend from 1 to 28 days of age. Both studies also found a significant and substantial reduction in UCP1 expression between days 7 and 28 days to a level that was barely detectable. Similar expression profiles were also found in the two studies for DIO2, PGC1α, PRLR and PRDM16 (a reduction from 1 to 28 days), and for PPARγ, adiponectin, HOXC9, and NR3C1 (an increase from 1 to 28 days), but the changes were not significant in the cited study. FABP4 showed a numerical increase from 1 to 28 days in both studies, though there was a peak at 7 days in the cited study, and the differences were not significant in either case. One gene, however, CIDEA, exhibited different expression profiles in the two studies. In the cited study, it was significantly downregulated by about 50 % between days 1 and 7, and again between days 7 and 28. In contrast, there was no significant difference between the three age groups in this study.

A more recent study looked at the global gene expression profiling of brown to white adipose tissue transformation in the perirenal depot of sheep, and used –2, 0, 0.5, 1, 2, 4 and 14 days of age as time points [Basse *et al*, 2015]. It found that the ages could be grouped into three distinct periods: a brown phase, comprising the –2 and 0 day time points; a transition phase comprising the 0.5, 1, 2 and 4 day time points; and a white phase, at 14 days of age. It tested eight genes in common with this study: the metabolic genes leptin and RIP140; the thermogenic genes DIO2, PGC1α and UCP1; and the developmental genes HOXC9, LHX8 and PRDM16. Most of the results were presented in terms of the three phases, so the trend from the transition phase (days 0.5 to 4) to the white phase (day 14) can be compared with the trend from 1 to 28 days in this study. The results were generally consistent with both those of this study (sternal) and those of Pope *et al* (perirenal). Expression of both leptin and RIP140 increased significantly from the transition phase to the white phase, while UCP1 expression was high and relatively stable until day 4, before declining to an almost undetectable level by day 14. DIO2 and PGC1α were highly expressed in the brown phase (not tested in this study), but their expression declined stepwise and more rapidly than that of UCP1, such that it was already much reduced by days 1 and 4, before reaching very low levels by day 14. The cited study also found significant increases in expression of HOXC9 and LHX8 between the transition and white phases. The only gene which differed

to any extent between the Basse *et al* study and this study was PRDM16, whose expression did not change in the cited study, but declined significantly by about 50 % from 1 to 28 days in this study (albeit at very low levels). It should be noted, however, that p = 0.0779 for the Kruskal-Wallis test, so no differences between groups would have been found (or *post hoc* tests performed) at the level of significance normally used in physiology (p < 0.05).

Overall, therefore, the hypothesis that the ontogeny of gene expression in the sternal adipose tissue of newborn lambs over the first month after birth would be similar to that of perirenal adipose tissue is supported. The most important difference is probably that of CIDEA, which encodes a protein that controls lipid droplet formation (Section 1.8.4.2, p 60) [Puri *et al*, 2008; Wu *et al*, 2014]. It is downregulated over the first month of postnatal life in perirenal adipose tissue, but its expression is unchanged in sternal adipose tissue. It is worth noting, however, that its expression profiles for the two individual sexes in this study were more dissimilar than those of most other genes (Appendix B, Figure B-4B, p 286). In males there was an upward trend with age, whereas in females there was a general downward trend with a peak at 7 days of age, although none of the differences were significant. It is of particular interest because it is highly expressed in the BAT of mice, and used as a marker of that tissue, but it is not expressed in their WAT [Zhou *et al*, 2003]. In contrast, it is highly expressed in the WAT of humans [Nordström *et al*, 2005]. Further work will be required to confirm its apparent tissue-specific, and possibly sex-specific, pattern of expression in the adipose tissue of sheep.

3.5.2.2 Expression of thermogenic genes in the sternal adipose tissue of sheep declines with age

The hypothesis that expression of thermogenic genes in the sternal adipose tissue of newborn lambs would reduce over the first month after birth is well supported. Expression of four of the genes tested, DIO2, PGC1α, PRLR and UCP1, declined substantially with age, which accords with a diminishing need for NST as the animals grow. Expression of ATF2 increased over the period, but that is probably a classification problem. It was categorised as thermogenic because it promotes the expression of the thermogenic genes PGC1α and UCP1 (Figure 1.3, p 26) [Cao *et al*, 2004; Collins *et al*, 2010], but it is a multifunctional gene that is involved in a number of other roles, including adipogenesis (Section 1.8.4.1, p 59) [Bhoumik *et al*, 2005; Bruhat *et al*, 2007; Lee *et al*, 2001]. Its increased expression is therefore probably the net result of a number of different influences. As discussed in Section 3.5.2.1, p 140, expression of CIDEA (Section 1.8.4.2, p 60) did not change with age in sternal adipose tissue, though it seems to decline with age in perirenal adipose tissue [Pope *et al*, 2014]. It is an established marker of BAT in rodents [Symonds, 2013], which is why it was classified as

thermogenic, although somewhat paradoxically it actually appears to inhibit NST [Zhou *et al*, 2003]. However, its pattern of expression is species-specific [Nordström *et al*, 2005; Zhou *et al*, 2003], and its role in sheep has yet to be fully elucidated.

3.5.2.3 The lineage of UCP1-expressing adipocytes in the sternal adipose tissue of sheep is unclear

As expected, there was no consistent pattern in the expression of genes classified as developmental. PRDM16 (Section 1.8.5.3, p 64), whose expression declined significantly from 1 to 28 days of age (subject to the proviso on the Kruskal-Wallis test noted in Section 3.5.2.1, p 140), determines the BAT fate of adipomyocite precursors (Figure 1.4, p 31) [Seale *et al*, 2008; Seale *et al*, 2007]. Its low absolute level of expression is consistent with its main role as a regulator of brown fat adipogenesis being fulfilled prior to birth, as BAT is recruited prenatally in precocial animals [Cannon & Nedergaard, 2004].

The role of HOXC9 (Section 1.8.5.1, p 63) in adipose tissue is unclear, but it was included in the study as it was once considered to be a marker of brite/beige adipose tissue [Waldén *et al*, 2012]. However, it is now considered to be a marker of both brite/beige adipose tissue and WAT [de Jong *et al*, 2015]. Indeed, the significant increase in its expression in the first month after birth in sternal adipose tissue is consistent with its status as a marker of WAT, and also accords with the findings of Pope *et al* [2014] and Basse *et al* [2015] in perirenal adipose tissue. Although expression of LHX8 (Section 1.8.5.2, p 64) increased significantly from 1 to 7 days and from 7 to 28 days, it should be noted that p = 0.0575 for the Kruskal-Wallis test, so no significant differences would have been revealed using stricter criteria (ie p < 0.5). Its role in adipose tissue is also unknown, but it is considered to be a marker of classical BAT [Petrovic *et al*, 2010; Seale *et al*, 2007; Timmons *et al*, 2007]. The increase in its expression over the first postnatal month, therefore, is inconsistent with the reduced thermogenic capacity and 'whitening' of the tissue. It was not one of the genes tested by Pope *et al*, but Basse *et al* also observed an increase in its expression over time in perirenal adipose tissue. Expression of SHOX2 (Section 1.8.5.4, p 65) did not change with age, though notably it was expressed at much higher levels (1 000–1 400 copies) than the other developmental genes tested (around 200 copies or less at any age). It was not tested in perirenal adipose tissue by either Pope *et al* or Basse *et al*. It has no role in adipogenesis, but it seems to be involved in determining the function, distribution and phenotype of adipose tissue in humans and mice, possibly through regulation of ADRB3 expression [Lee *et al*, 2013]. It is highly expressed in the subcutaneous adipose tissue of humans and mice, but minimally expressed in visceral adipose tissue and WAT, and has therefore been considered to be a

marker of brite/beige adipose tissue [Lee *et al*, 2013; Waldén *et al*, 2012]. However, if it were such a marker, its expression would be expected to decline with age.

Overall, therefore, results are inconclusive, and there is insufficient evidence to either support or reject the hypothesis that UCP1-expressing adipocytes in sternal adipose tissue would be brite/beige rather than brown. Indeed, recent evidence suggests that, at least in mice, most of the commonly-cited markers are uninformative with regard to adipocyte type classification [de Jong *et al*, 2015].

3.5.2.4 Expression of some adipogenic genes in sternal adipose tissue over the first month of postnatal life is inversely correlated

The hypothesis that the expression of adipogenic and metabolic genes in sternal adipose would increase in the first month after birth is not so well supported. While most genes did show increased expression, there were a few notable exceptions: expression of FABP4 and insulin receptor (INSR) did not change, expression of C/EBPα peaked at 7 days of age and then reverted to a 1 day level, and expression of SREBF1 declined with age. As discussed in Section 3.5.2.1, p 140, gene expression profiles were similar to those reported in perirenal adipose tissue where the same genes were tested [Basse *et al*, 2015; Pope *et al*, 2014]. FABP4 (Section 1.8.3.2, p 56) is often used as a marker of adipocyte differentiation [Shan *et al*, 2013], so the fact that its expression did not increase significantly may suggest that the growth in sternal adipose tissue mass is more a function of hypertrophy than hyperplasia. Histological analysis will be required to support or reject this hypothesis. More curious, however, is the contrasting expression profiles of some adipogenic genes. PPARγ (Section 1.8.2.3, p54) and C/EBPα (Section 1.8.2.1, p 53) are the principal regulators of adipogenesis and promote each other's expression (Figure 1.5, p 34) [Rosen *et al*, 2002]. Their expression is therefore normally positively correlated, and no instances could be found in the literature where that relationship is reversed. Similarly, expression of SREBF1 (Section 1.8.2.4, p 55), would be expected to correlate with that of PPARγ and C/EBPα, although the role of its principal protein isoform, SREBP1c, in adipogenesis is less well-defined than that of the two main regulators [White & Stephens, 2010]. The expression profiles for the combined sexes (PPARγ increasing with age, C/EBPα peaking at 7 days of age, and SREBF1 declining with age) are mirrored by the individual sexes (Appendix B, Figure B-2, p 284), albeit with significance reduced or lost, which gives confidence that the effect is genuine. However, expression of neither C/EBPα nor SREBF1 was tested by Pope *et al* or Basse *et al* in perirenal adipose tissue, so it is unclear whether this is a general or tissue-specific effect. Further work will therefore be required to confirm the effect in sternal adipose tissue, and to ascertain whether it is replicated in perirenal and other adipose tissues.

3.6 Summary and conclusions

A study was carried out to investigate changes in gene expression in the sternal adipose tissue of sheep over the first month of postnatal life. Although similar studies have been undertaken in perirenal adipose tissue, no such studies in sternal adipose tissue have been previously reported. It was hypothesised that gene expression profiles would be similar to those of perirenal adipose tissue, that there would be a reduction in expression of thermogenic genes with age, that there would be an increase in expression of adipogenic and metabolic genes with age, and that UCP1-expressing adipocytes would be of brite/beige rather than classical brown lineage.

It was found that the pattern of gene expression in sternal adipose tissue was similar to that of perirenal adipose tissue, with the main difference being for CIDEA, which encodes a protein involved in lipid droplet formation [Puri *et al*, 2008; Wu *et al*, 2014]. Its expression declined with age in perirenal adipose tissue, but was unchanged in sternal adipose tissue. The expression of almost all thermogenic genes declined substantially with age to barely detectable levels, which is consistent with the diminishing requirement for NST as animals grow in size. The main exception, again, was CIDEA, which is a marker of BAT in mice, but which is highly expressed in WAT in humans [Nordström *et al*, 2005; Zhou *et al*, 2003]. Its expression pattern therefore seems to be species-specific, and its role in sheep is yet to be fully determined. While expression of most metabolic genes increased with age, expression of adipogenic genes was more varied. In particular, there was a disparity in the expression of the two main regulators of adipogenesis, PPARγ and C/EBPα. Expression of the former increased with age, while that of the latter peaked at 7 days of age and then declined to a 1-day level. As these two genes promote each other's expression [Rosen *et al*, 2002], the result was surprising. Expression of another important adipogenic gene, SREBF1, which would be expected to correlate with that of PPARγ and C/EBPα, declined with age. Further work will be required to confirm these unexpected expression patterns in sternal adipose tissue, and to ascertain whether they are replicated in other adipose tissues. Finally, no conclusions could be drawn about the lineage of UCP1-expressing adipocytes in sternal adipose tissue, as expression of HOXC9 and LHX8, originally considered respectively to be markers of WAT or brite/beige adipose tissue and classical BAT, increased with age, while that of SHOX 2, considered to be a marker of brite/beige adipose tissue, did not change. A recent report has found that these so-called markers are not generally informative as to adipocyte type [de Jong *et al*, 2015].

4 The effect of maternal fat supplements on the sternal adipose tissue of suckling lambs

4.1 Introduction

4.1.1 Overview

This study (Study A) examines the effect of fat supplements fed to lactating ewes on the expression of adipogenic, metabolic, thermogenic and developmental genes in the sternal adipose tissue of their 7 and 28 day-old suckling lambs. The fat supplements used were sunflower and canola oil, which like all vegetable oils, are a mixture of different fatty acids.

4.1.2 Fatty acids

Fatty acids are long hydrocarbon chains of various lengths and degrees of unsaturation that end in a carboxyl (COOH) group [Berg *et al*, 2012]. They are deemed to be 'saturated' when all the carbon-carbon bonds are single bonds and 'unsaturated' when one or more of the carbon-carbon bonds is a double or triple bond.

4.1.2.1 Nomenclature

A common system of nomenclature for fatty acids is of the form '*x:y n-z*', where *x* is the number of carbon atoms, *y* is the number of double bonds, and *z* is the position of the first double bond, counting carbon atoms from the methyl (CH_3) end of the carbon chain. The '*n-z*' group is omitted if *y* is zero (ie the fatty acid is saturated, and therefore has no double bonds). For example, the saturated fatty acid stearic acid is coded 18:0, indicating that it has 18 carbon atoms and no double bonds. Similarly, the monounsaturated fatty acid oleic acid is coded 18:1 *n*-9, indicating that it has 18 carbon atoms and one double bond starting at the ninth carbon atom. The letter *n* (usually italicised) that prefixes the position of the first double bond is synonymous with the more common term 'omega' (ω), which is used to describe different classes of polyunsaturated fatty acids that are vital for human health.

4.1.2.2 Essential fatty acids

The most important ω-3 fatty acids are α-linolenic acid (ALA; 18:3 *n*-3), eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:6 *n*-3), while the most important ω-6 fatty acids are linoleic acid (LA; 18:2 *n*-6) and arachidonic acid (ARA; 20:4 *n*-6). EPA and DHA are mainly obtained from fish and seafood, ARA from meat and poultry, and LA and ALA from plant sources such as vegetables, vegetable oils and cereals [Meyer *et al*, 2003]. Mammals lack the necessary desaturase enzymes to create any double bonds closer to the methyl end of the chain than the ninth carbon atom, so ω-3 and ω-6 fatty acids must be obtained from the diet, and are therefore often termed 'essential' fatty acids. However, the term is usually applied more specifically to the 'base' ω-3 and ω-6 fatty acids, ALA and LA, as the more complex ω-3 and ω-6 fatty acids can be synthesised in the body from these precursors by a process of elongation and desaturation [Barceló-Coblijn & Murphy, 2009].

The ratio of ω-6 to ω-3 fatty acids, particularly LA to ALA, also has an impact on health, as they compete for the same desaturase, elongase and other enzymes [Barceló-Coblijn & Murphy, 2009; Lorente-Cebrián *et al*, 2013]. Increased consumption of high LA-containing grains in the western diet has raised the LA:ALA ratio from 8:1 to up to 20:1, far exceeding the optimal figure of around 4:1 [Barceló-Coblijn & Murphy, 2009]. Such an imbalance may favour conversion of LA to ARA over conversion of ALA to EPA/DHA [Ailhaud *et al*, 2006].

4.1.3 Canola

Canola is a plant that was originally bred from rapeseed, but which has a very different chemical composition and nutritional profile [Canola Council of Canada, 2015]. It was developed because rapeseed has a high percentage (around 50 %) of erucic acid (22:1 *n*-9), which has pathological effects in humans and animals [Thomasson & Boldingh, 1955]. Its name is a contraction of 'Canada' and 'ola', meaning oil. The name is regulated, and one of the requirements for its use is that erucic acid should make up less than 2 % of its fatty acid content [Canola Council of Canada, 2015].

4.1.4 Fatty acid composition of sunflower and canola oils

The fatty acid composition of specific vegetable oils is highly variable, and this is reflected in the ranges set by the Food and Agriculture Organization (FAO) of the United Nations in their standards for vegetable fats and oils [FAO, 1999]. For example, the content of oleic acid is set at 51.0–70.0 % for canola oil and 14.0–39.4 % for sunflower oil,[32] while that of LA

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[32] Canola oil is listed as 'Rapeseed oil (low erucic acid)'.

Table 4.1 Fatty acid composition of sunflower and canola oils. The percentages shown are representative and are based on tables published by Dubois *et al* [2007]. There is a wide variation in the fatty acid content of vegetable oils and in the permissible range for each constituent fatty acid set by the Food and Agriculture Organization of the United Nations [FAO, 1999]. SFA is saturated fatty acid; MUFA is monounsaturated fatty acid; PUFA is polyunsaturated fatty acid.

Fatty acid	Formula	Sunflower %	Canola %
Palmitic acid	16:0	6	5
Stearic acid	18:0	5	2
Other SFAs		1	1
Total SFAs		12	8
Oleic acid	18:1 *n*-9	22	60
Other MUFAs		–	1
Total MUFAs		22	61
Linoleic acid	18:2 *n*-6	66	21
α-linolenic acid	18:3 *n*-3	–	10
Total PUFAs		66	31

is set at 15.0–30.0 % for canola oil and 48.3–74.0 % for sunflower oil. The actual fatty acid composition of the two supplements used in the study is not known, but typical values are shown in Table 4.1, above. Irrespective of their specific contents, the important differences between the two supplements are that (1) canola oil has a relatively high proportion of ω-9 and a relatively low proportion of ω-6 fatty acids, whereas the figures are reversed with sunflower oil, and (2) sunflower oil has no ω-3 fatty acids, while canola oil has around 10 % (the FAO standards for ALA being 5.0–14.0 % for canola oil and 0-0.3 % for sunflower oil).

4.2 Hypotheses

It was hypothesised that the offspring of ewes receiving a fat supplement would increase their adipose tissue mass relative to controls, and that this would be a function of both hypertrophy and hyperplasia. It was therefore expected that expression of genes involved in adipogenesis and metabolism would increase in the offspring of supplemented ewes relative to controls. The effect of maternal supplements on the expression of thermogenic genes in young lambs was more difficult to predict, but it was hypothesised that there would be a compensatory mechanism whereby the predicted increase in fat mass as a result of supplementation would be partially offset by an increase in thermogenic activity.

The hypotheses for this chapter, therefore, were that fat supplements fed to lactating ewes would, in respect of their suckling lambs:

- increase their adipose tissue mass;

- increase the expression of adipogenic, metabolic and thermogenic genes in their sternal adipose tissue.

4.3 Materials and methods

4.3.1 Animal husbandry and interventions

4.3.1.1 Overview

Ewes were allocated to one of three feeding groups, one control and two supplemented, for 28 days after parturition, and their suckling lambs were sampled at 7 and 28 days of age. All experimental procedures were undertaken in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986, and with the approval of the appropriate Ethics Committee of the University of Nottingham.

4.3.1.2 Source, location and housing

A flock of 28 pregnant Bluefaced Leicester cross Swaledale mule ewes was obtained from a commercial farm near Skipton in North Yorkshire. The ewes had been mated with a Texel ram. Two weeks before the first ewes were due to lamb, the flock was transported to a barn at the Melton Lane Complex on the University of Nottingham's Sutton Bonington campus. The ewes were housed together in a single pen until they gave birth, at which point they were moved with their offspring to individual pens of about 2 m × 2 m.

4.3.1.3 Allocation of ewes to feeding groups

Ewes were allowed to lamb normally at term. The first ewe gave birth on 4 March 2013 and the last on 5 April 2013. Of the 28 ewes, 23 gave birth to twins and five to triplets, yielding an initial total of 61 lambs: 26 males (M) and 35 females (F). Immediately after parturition, ewes were allocated to one of three nutritional groups: standard or 'normal' nutrition (N; n = 10); standard nutrition with a sunflower oil supplement (S; n = 9); or standard nutrition with a canola oil supplement (C; n = 9) (Figure 4.1, p 150). Allocations were made so as to ensure, as far as possible, that each feeding group had an even distribution of offspring by sex.
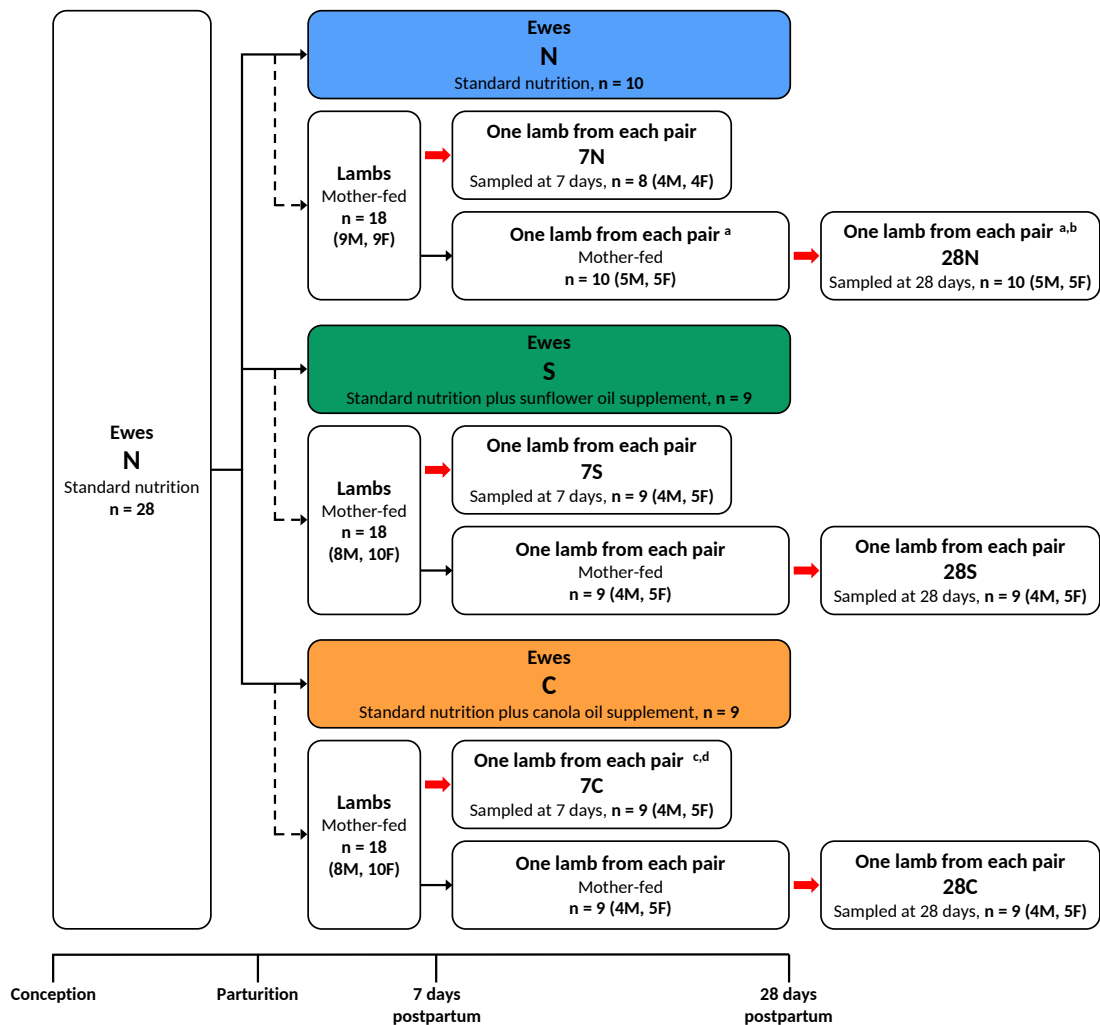
Figure 4.1 Experimental protocol. Pregnant ewes were fed a standard diet until parturition, and were allowed to lamb normally at term (21 weeks). Immediately after parturition, the ewes were allocated to one of 3 feeding groups: N, standard or 'normal' nutrition; S, standard nutrition with a 3 % supplement of sunflower oil; and C, standard nutrition with a 3 % supplement of canola oil. Allocations were made so as to ensure, as far as possible, that each feeding group of ewes had an even distribution of offspring by sex. All ewes remained on their allocated diets until they were separated from the last of their offspring. The lambs were fed by their mothers until they were sampled. One lamb from each ewe was humanely euthanased and sampled at around 7 (± 1) days of age and the other at around 28 (± 3) days of age. Allocation of each pair of lambs to the 2 sampling time points was random for MM and FF pairs, but in the case of MF pairs was influenced by the need to maintain a sex balance within each age group. M is male and F is female. The colour coding is intended to facilitate interpretation of the results figures in this chapter.

a   One ewe had a twin lamb which was stillborn and another had a twin lamb which died within 24 hours of birth. The surviving siblings, both, males, were retained in the experiment and sampled at 28 days.

b   One of the singleton lambs had stunted growth after birth, and it was determined that its mother was not producing enough milk. This lamb has therefore been excluded from further analysis.

c   One lamb was growth restricted at birth and weighed over 1 kg less than the next smallest animal. This lamb has also been excluded from further analysis.

d   One lamb was found to have spinal damage, an enlarged heart, and a haematoma near the gut cavity. This lamb has also been excluded from further analysis.

4.3.1.4 Number and classification of lambs

One female lamb (from an MF twin pair) was stillborn. The 60 remaining lambs (26M, 34F) were weighed as soon as possible after birth. Another female lamb (also from an MF twin pair) died within a day of birth, leaving 59 surviving lambs (26M, 33F). The two surviving male siblings of the dead females (both from ewes in group N) were retained in the study as unmatched singletons. One lamb from each set of triplets (1M, 4F) was removed from its mother on the day after birth for tissue sampling,[33] leaving its two siblings as a pseudo-twin pair. The 54 remaining lambs (25M, 29F) have been classified according to the feeding group of their mothers: N (n = 18; 9M, 9F); S (n = 18; 8M, 10F); or C (n = 18; 8M, 10F) (Figure 4.1, p 150).

4.3.1.5 Feeding

All ewes were fed the same diet until parturition, comprising 2 kg hay and 1 kg commercial concentrate (Ewe 18 Mix; Manor Farm Feeds, Oakham, Leicestershire, UK), divided into two meals, one in the morning and one in the afternoon. The concentrate was designed for pregnant and lactating ewes, and was sufficient to fulfil recommended energy and protein requirements [Alderman & Cottrill, 1993]. In its standard form, it contained 3 % crude oils and fats. After parturition, ewes allocated to the N group continued on the same diet. Ewes allocated to the S and C groups were fed 1 kg of a supplemented concentrate in place of the standard form. This contained a further 3 % of either sunflower oil or canola oil, thereby doubling the total amount of fats and oils. The analytical constituents of the concentrates are shown in Table 4.2, below. All ewes also received water *ad libitum*, and remained on

Table 4.2 Analytical constituents of concentrate by feeding group. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C).

Item	N %	S %	C %
Canola oil	0	0	3
Sunflower oil	0	3	0
Other crude oils and fats	3	3	3
Crude protein	18	18	18
Crude fibre	7	7	7
Crude ash	6	6	6
Moisture	15	15	15

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[33] These 1 day-old lambs were used for the ontogeny study in Chapter 3.

their allocated diets until they were separated from the last of their offspring after 28 days. Lambs were milk-fed by their mothers until they were sampled.

4.3.1.6 Blood sampling

Blood was sampled from lambs just before they were euthanased. Each animal was shaved around the front of its neck, and up to 20 ml of blood was taken from the jugular vein with a 10 ml syringe in two extractions. The syringes were discharged into two collection tubes, a 10 ml EDTA tube (Vacutainer; BD, Plymouth, Devon, UK) and a 9 ml lithium heparin tube (S-Monovette; Sarstedt, Nümbrecht, Germany), which were immediately put on ice. As soon as possible after collection (ie within 20 min), the collection tubes were centrifuged at 4 °C and 1 200 xg for 15 min. The plasma was then extracted from each tube and dispensed into 2 ml cryovials for subsequent local storage at –20 °C. Within a week of collection, the cryovials of plasma were transferred to central storage at –80 °C. They were ultimately despatched on dry ice to Professor Duane Keisler, University of Missouri, USA, for analysis of metabolites.

4.3.1.7 Rectal temperatures

The rectal temperature of the lambs was taken once, just before they were euthanased, using an Eco Temp MC-246-E digital thermometer (Omron, Milton Keynes, UK).

4.3.1.8 Tissue sampling

One lamb from each ewe was sampled at around 7 ($\pm$ 1) days of age and the other at around 28 ($\pm$ 3) days of age. Allocation of each pair of lambs to the two sampling time points was random for MM and FF pairs, but in the case of MF pairs was influenced by the need to maintain a sex balance within each age group. The two unmatched singleton males were both sampled at 28 days of age. The prefix '7' or '28' has been used with the feeding group identifier to distinguish the 6 age/feeding group combinations: 7N (n = 8; 4M, 4F); 7S (n = 9; 4M, 5F); 7C (n = 9; 4M, 5F); 28N (n = 10; 5M, 5F); 28S (n = 9; 4M, 5F); 28C (n = 9; 4M, 5F).

The lambs were weighed before sampling, then humanely euthanased by an injection of an overdose of barbiturate (200 mg kg^{-1} BW pentobarbital sodium; Euthatal; Merial Animal Health, Harlow, Essex, UK) into the jugular vein. Carcasses were promptly dissected, and all major tissues and organs, including perirenal, pericardial, omental and sternal adipose tissue, were excised and rapidly weighed. Samples from each organ and tissue were then snap-frozen in liquid nitrogen and stored at –80 °C. Samples from each adipose tissue depot were also preserved in formal saline (10 % formalin, 0.9 % sodium chloride) for subsequent

histology. All but seven of the dissections were carried out by Professor Mike Symonds, assisted by the author and a colleague, Mr Graeme Davies. Two 7 day-old animals were dissected by Dr Viv Perry, and five 28 day-old animals were dissected by the author.

4.3.1.9 Exclusions

Three lambs were subsequently excluded from further analysis. One female lamb (7C) was considered to be growth restricted, as it had a birth weight of only 2.52 kg, compared to a mean of 4.96 kg and a next lowest of 3.77 kg. One of the unmatched singleton male lambs (28N) had stunted growth, and it was determined that its mother had not been producing enough milk. Another male lamb (7C) was found to have spinal damage, with an enlarged heart and a haematoma near the gut cavity. The results therefore include data for 51 lambs (23M, 28F), 24 of which (11M, 13F) were sampled at 7 days and 27 of which (12M, 15F) were sampled at 28 days. The adjusted numbers for each group are: 7N (n = 8; 4M, 4F); 7S (n = 9; 4M, 5F); 7C (n = 7; 3M, 4F); 28N (n = 9; 4M, 5F); 28S (n = 9; 4M, 5F); 28C (n = 9; 4M, 5F).

4.3.1.10 Birth weights

Mean birth weights for each group are shown in Figure 4.2, below.
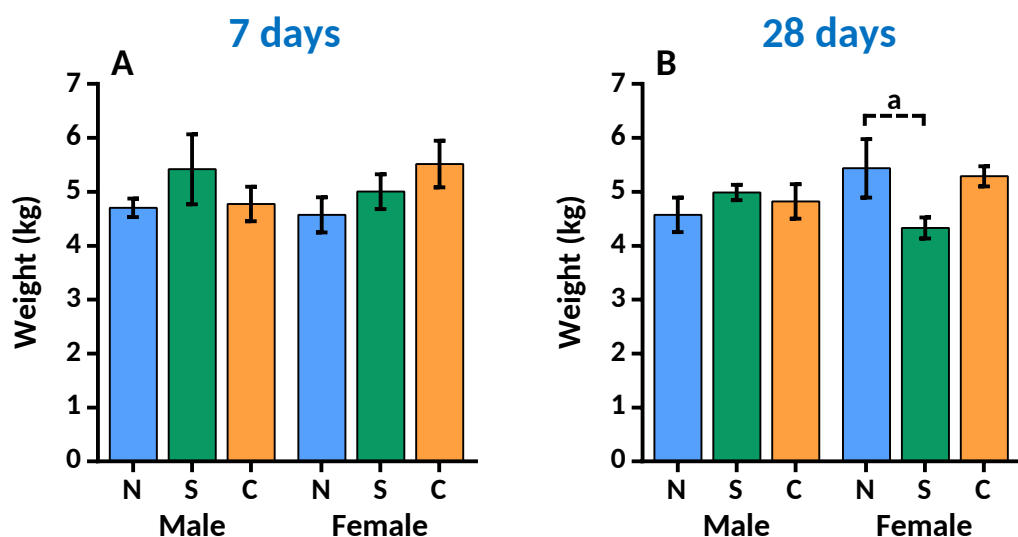
Figure 4.2 Birth weights. Values shown are mean birth weights ± SEM by feeding group and sex of lambs sampled at (**A**) 7 days and (**B**) 28 days. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). For the 7-day animals, n = 4 for all groups except CM (n = 3) and SF (n = 5); M is male, F is female. For the 28-day animals, n = 4 for male groups and n = 5 for female groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0952; p > 0.1 in all other cases).

4.3.2 Laboratory procedures

The laboratory procedures for determining gene expression are described in Section 2.4, p 70, the procedures for determining protein expression are described in Section 2.5, p 113, and histology procedures are described in Section 2.6, p 122.

4.3.3 Statistics

The overall methodology for statistical analysis and presentation of data is described in Section 2.7, p 128. In analysing results relating to the different feeding groups, statistical comparisons were usually made between each of the two supplemented groups (sunflower or canola) and controls. The underlying purpose of the study was to examine the effect of dietary supplementation, so a direct comparison between supplemented groups was of no particular interest. In one instance (Section 4.4.4, p 161), a comparison between males and females in one nutritional group was also made following a specific observation.

In determining effect sizes for statistical power calculations on tests of tissue weights, plasma metabolites and gene expression, the absolute percentage differences between the mean of each supplemented group (S or C) and the control group (N), and the coefficients of variation for each group, were averaged across all tissues, metabolites or genes.

A single estimate of required sample size has been carried out *post hoc* to cover all tests. For this purpose, a 'reasonable' effect size figure of 1.0 was used. This was calculated by assuming a difference between group means of 40 % and a coefficient of variation of 40 %. A larger difference between means and/or a smaller coefficient of variation would result in a smaller required sample size for the same power.

4.4 Results

4.4.1 Body and tissue weights

4.4.1.1 Body weights

As there was some variation in mean birth weights between groups (Figure 4.2, p 153) and some variation in age at sampling (7 ± 1 days or 28 ± 3 days), gains in weight to each age are shown rather than absolute body weight, and are presented in terms of the gain per day per kg birth weight. Mean weight gains per day per kg birth weight for each group are shown in Figure 4.3, p 155. The mean weight gain in the sunflower (S) female group was significantly higher than that of controls (N) to seven days (p = 0.0350), and there was weak evidence of a similar effect to 28 days (p = 0.0556).

Mean body weights at sampling for each group are shown in Table 4.3, below. Mean birth weights by feeding group and sex (but not sampling age) and mean body weights at 7 days by feeding group and sex for all lambs, including those sampled at 28 days, are shown in Appendix C, Table C-1, p 288. There were no significant differences between supplemented groups and controls in any case (p > 0.1).
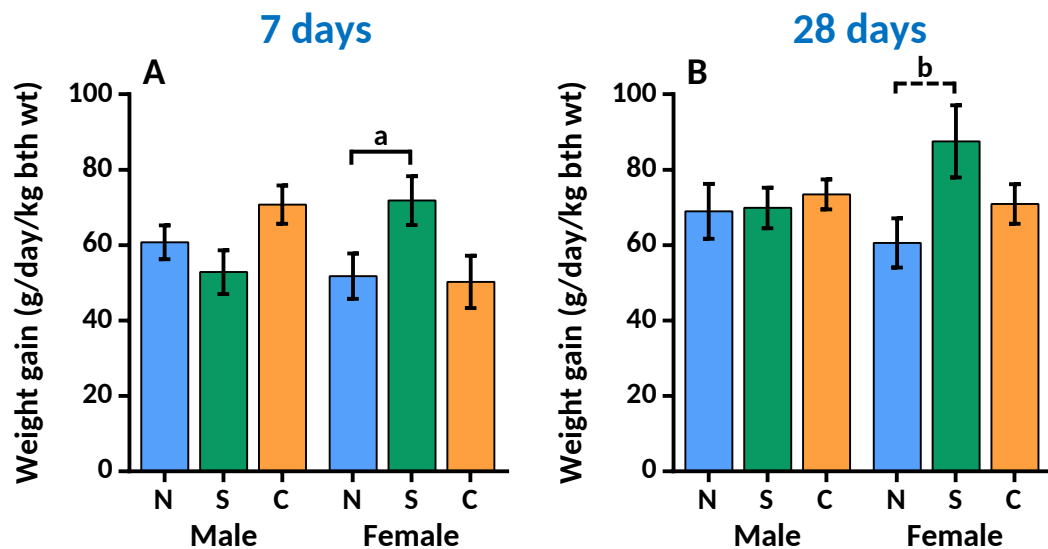
Figure 4.3 Body weight gain. Values shown are mean body weight gain per day per kg birth weight ± SEM by feeding group and sex to (**A**) 7 days and (**B**) 28 days. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). To 7 days, n = 7–8 for male groups and n = 8–10 for female groups. To 28 days, n = 4 for male groups and n = 5 for female groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0350; b: p = 0.0556; p > 0.1 in all other cases).

Table 4.3 Body weights at sampling. Values shown are mean body weights ± SEM by feeding group and sex of lambs sampled at 7 (± 1) and 28 (± 3) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). M is male, F is female. At 7 days, n = 4 for all groups except CM (n = 3) and SF (n = 5). At 28 days, n = 4 for M groups and n = 5 for F groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (p > 0.1 in all cases).

Age	Sex	Weight (kg)		
		N	S	C
7 days	M	6.90 ± 0.11	7.04 ± 0.64	6.96 ± 0.27
	F	6.42 ± 0.28	7.51 ± 0.55	7.26 ± 0.43
28 days	M	13.3 ± 0.6	15.1 ± 0.6	14.9 ± 1.1
	F	14.6 ± 0.9	14.7 ± 0.7	15.7 ± 0.3

4.4.1.2 Tissue weights

Absolute weights of omental, pericardial, perirenal and sternal adipose tissue, and their absolute combined weight, at 7 days of age are shown in Table 4.4, p 157. The equivalent values relative to body weight (BW) are shown in Table 4.5, p 157. Absolute and relative weights at 28 days of age are shown in Figure 4.4, p 158, and Figure 4.5, p 159, respectively.

There were no significant differences in tissue weights between supplemented groups and controls in male lambs in absolute or relative terms at 7 days (p > 0.1 in all cases). In female lambs, the absolute weight of sternal adipose tissue in the sunflower (S) group at 7 days was significantly higher than that of controls (N; p = 0.0317); however, this difference disappeared when the sternal adipose tissue weight was expressed relative to body weight (p > 0.1). There were no other significant differences between supplemented groups and controls in female lambs in absolute or relative terms at 7 days (p > 0.1 in all cases).

There were no significant differences in tissue weights between supplemented groups and controls in male lambs in absolute or relative terms at 28 days (p > 0.1 in all cases). In female lambs at 28 days, however, there were significant differences in tissue weights between one or both supplemented groups and controls in absolute terms for all tissues except pericardial. The absolute weight of tissue in the sunflower group was higher than that of controls for omental (p = 0.0317), perirenal (p = 0.0317) and combined (p = 0.0159) adipose tissue, while that of the canola group (C) was higher than that of controls for sternal (p = 0.0079) and combined (p = 0.0317) adipose tissue. In general, adjustment for body weight reduced the value of p for the NS comparison and increased it for the NC comparison. In relative terms at 28 days, therefore, the weight of tissue in the S group remained significantly higher than that of controls for omental (p = 0.0159), perirenal (p = 0.0317) and combined (p = 0.0079) adipose tissue, and there was weak evidence for a similar effect with pericardial (p = 0.0952) and sternal (p = 0.0952) adipose tissue. However, there were no significant differences in tissue weights in relative terms at 28 days between the C group and controls, although there was weak evidence that the relative weight of sternal adipose tissue in the C group was higher than that of controls (p = 0.0556).

4.4.2 Rectal temperatures

Rectal temperatures were taken from lambs immediately before they were euthanased at around 7 or 28 days of age, except in the case of one 28 day-old female from the canola group (where the reading was omitted for reasons which were not recorded). Mean rectal temperatures are shown in Table 4.6, p 160. The rectal temperature of 7-day old males in the sunflower group (S) was significantly, if not substantially, lower than that of controls

Table 4.4 Absolute adipose tissue weights at 7 days. Values shown are mean tissue weights ± SEM by depot, sex and feeding group of lambs sampled at 7 (± 1) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); M is male, F is female; n = 4 for all groups except CM (n = 3) and SF (n = 5). S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0317; p > 0.1 in all other cases).

Item	Sex	Adipose tissue weight (g)		
		N	S	C
Omental	M	16.2 ± 2.0	11.2 ± 2.7	15.9 ± 2.5
	F	13.4 ± 2.1	15.0 ± 4.7	16.3 ± 4.4
Pericardial	M	8.49 ± 0.32	10.05 ± 2.73	9.35 ± 0.61
	F	6.69 ± 0.93	8.53 ± 1.12	8.10 ± 1.85
Perirenal	M	40.9 ± 3.0	41.6 ± 4.4	48.5 ± 6.3
	F	46.7 ± 6.3	64.1 ± 11.0	55.7 ± 10.4
Sternal	M	14.9 ± 1.4	13.0 ± 3.3	19.6 ± 5.5
	F	16.7 ± 1.9 [a]	23.2 ± 1.9 [a]	20.0 ± 5.7
Combined	M	80.5 ± 3.3	75.8 ± 8.8	93.3 ± 14.0
	F	83.4 ± 9.3	111.0 ± 16.6	100.0 ± 20.1

Table 4.5 Relative adipose tissue weights at 7 days. Values shown are mean tissue weights ± SEM relative to body weight (BW) by depot, sex and feeding group of lambs sampled at 7 (± 1) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); M is male, F is female; n = 4 for all groups except CM (n = 3) and SF (n = 5). S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (p > 0.1 in all cases).

Item	Sex	Adipose tissue weight (g per kg BW)		
		N	S	C
Omental	M	2.33 ± 0.25	1.53 ± 0.28	2.28 ± 0.33
	F	2.07 ± 0.29	1.97 ± 0.57	2.18 ± 0.54
Pericardial	M	1.23 ± 0.06	1.51 ± 0.52	1.34 ± 0.05
	F	1.03 ± 0.11	1.13 ± 0.10	1.09 ± 0.21
Perirenal	M	5.94 ± 0.48	5.98 ± 0.68	6.93 ± 0.62
	F	7.22 ± 0.82	8.33 ± 0.92	7.70 ± 1.51
Sternal	M	2.16 ± 0.20	1.93 ± 0.62	2.77 ± 0.67
	F	2.60 ± 0.26	3.15 ± 0.33	2.80 ± 0.89
Combined	M	11.7 ± 0.4	10.9 ± 1.6	13.3 ± 1.5
	F	12.9 ± 1.1	14.5 ± 1.4	13.8 ± 2.9
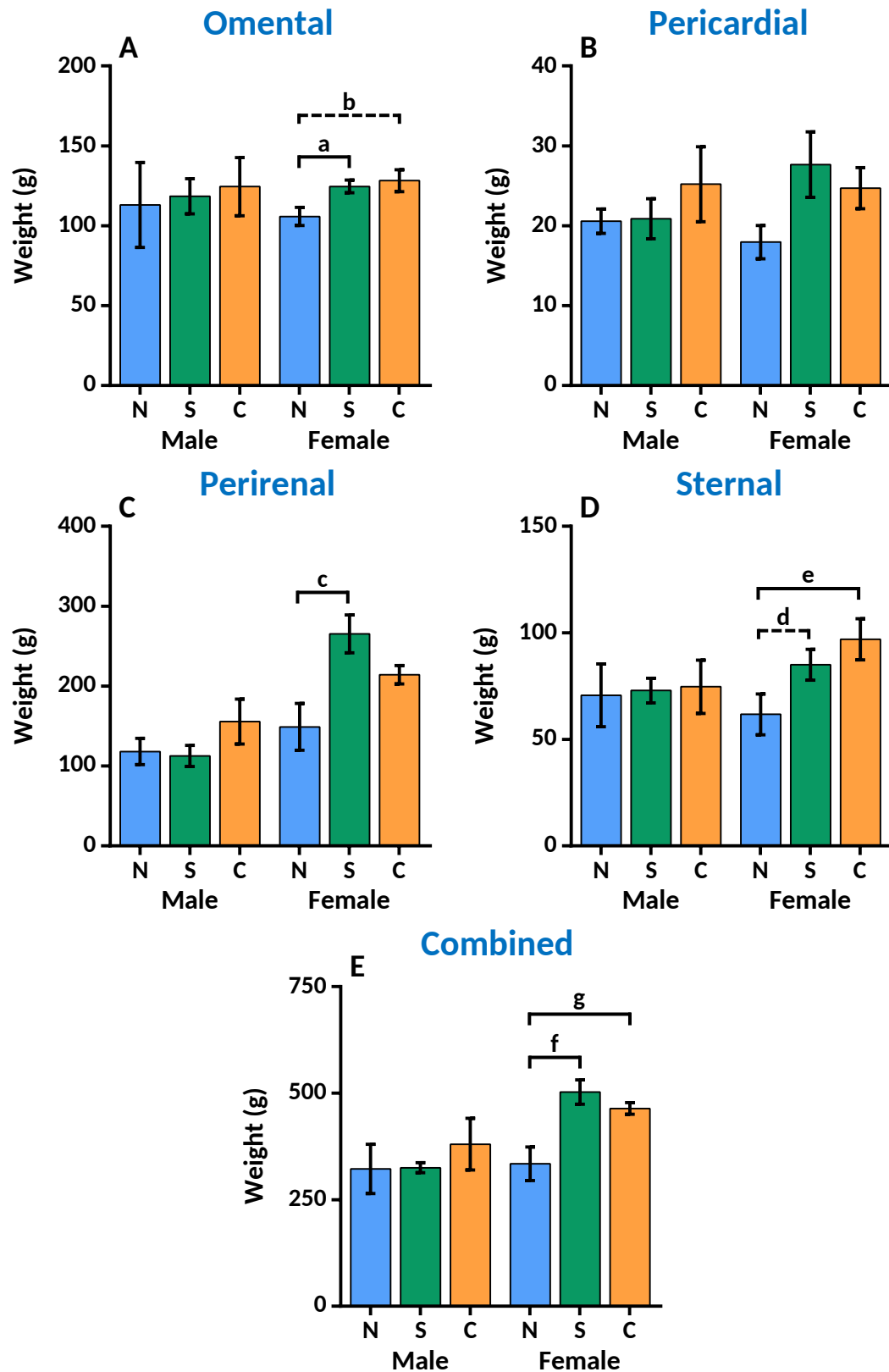
Figure 4.4 Absolute adipose tissue weights at 28 days. Values shown are mean tissue weights ± SEM by depot, feeding group and sex of lambs sampled at 28 (± 3) days of age. The 'combined' figure (**E**) is the total adipose tissue from the four depots weighed. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for male groups and n = 5 for female groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0317; b: p = 0.0556; c: p = 0.0317; d: p = 0.0556; e: p = 0.0079; f: p = 0.0159; g: p = 0.0317; p > 0.1 in all other cases).
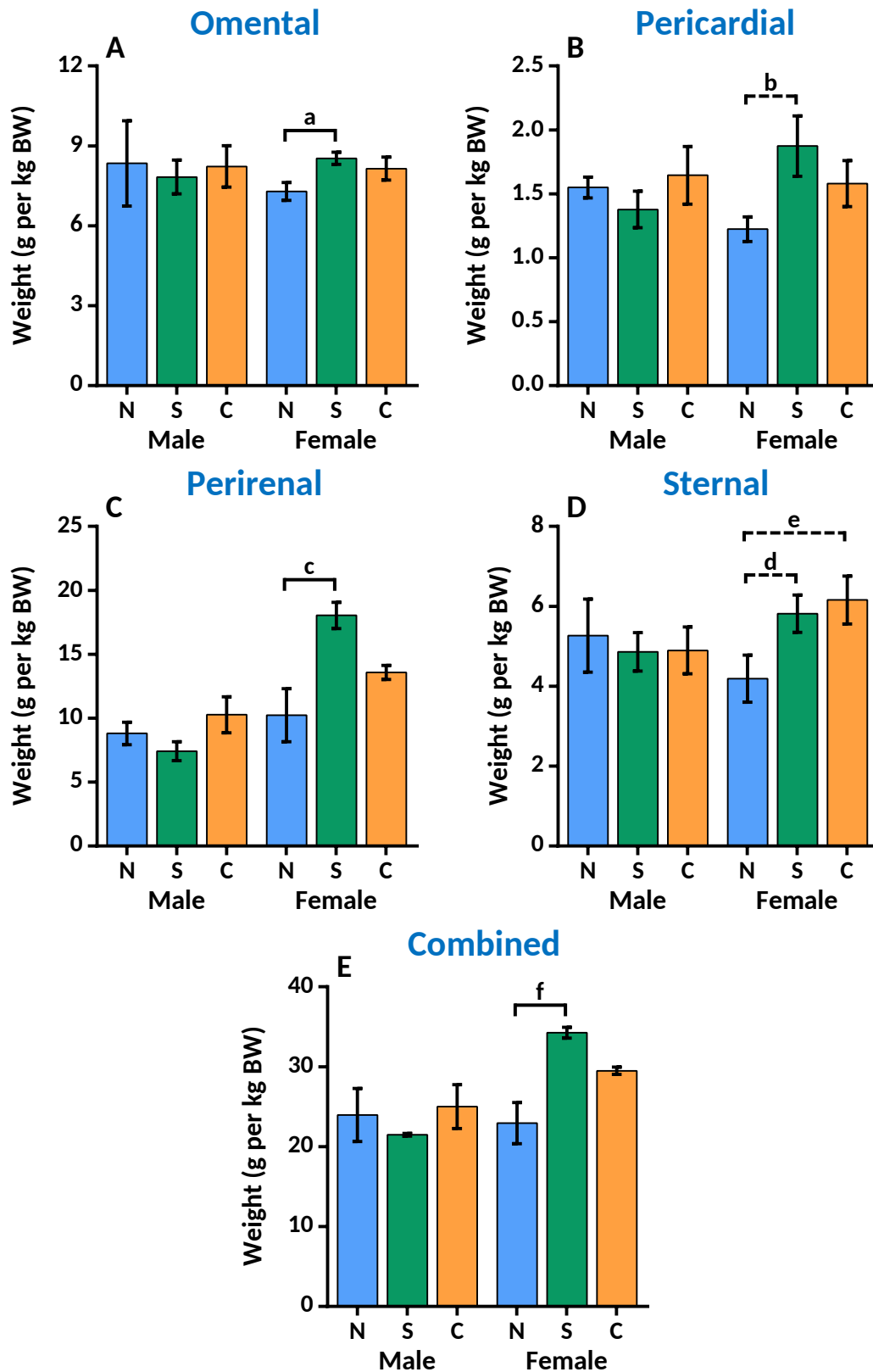
Figure 4.5 Relative adipose tissue weights at 28 days. Values shown are mean tissue weights ± SEM relative to body weight (BW) by depot, feeding group and sex of lambs sampled at 28 (± 3) days of age. The 'combined' figure (**E**) is the total adipose tissue from the four depots weighed. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for male groups and n = 5 for female groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0159; b: p = 0.0952; c: p = 0.0317; d: p = 0.0952; e: p = 0.0556; f: p = 0.0079; p > 0.1 in all other cases).

Table 4.6 Rectal temperatures by age, sex and feeding group. Values shown are mean rectal temperatures ± SEM for lambs just before they were sampled at 7 (± 1) and 28 (± 3) days. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). M is male, F is female. At 7 days, n = 4 for all groups except CM (n = 3) and SF (n = 5). At 28 days, n = 4 for all groups except NF and SF (n = 5). S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0286; p > 0.1 in all other cases).

Age	Sex	Temperature (°C)		
		N	S	C
7 days	M	40.58 ± 0.14 [a]	40.10 ± 0.05 [a]	40.25 ± 0.17
	F	40.24 ± 0.17	40.35 ± 0.07	40.26 ± 0.10
28 days	M	40.17 ± 0.07	40.09 ± 0.13	40.18 ± 0.13
	F	39.89 ± 0.12	39.96 ± 0.13	40.09 ± 0.11

(N; p = 0.0286). The difference in mean temperature was about 0.5 °C (40.58 ± 0.14 versus 40.10 ± 0.05). There were no other significant differences between supplemented groups and controls in either males or females at 7 or 28 days of age (p > 0.1 in all cases).

4.4.3 Plasma metabolites

Blood was sampled from lambs immediately before they were euthanased at around 7 or 28 days of age. Unfortunately, there were only two male and three female lambs in the 7-day canola (C) group for plasma analysis. As well as the two animals from that group (1F, 1M) which were excluded from the study for restricted growth and spinal damage respectively, a further two animals (1M, 1F) did not immediately yield sufficient blood for analysis, and the extraction was abandoned to avoid stressing them. All the remaining 7 and 28 day-old groups comprised 4 or 5 animals of each sex.

The mean plasma concentrations of IGF1 are shown in Table 4.7, p 161. There were no significant differences between the sunflower (S) or canola (C) groups and controls (N) at either 7 or 28 days, although there was weak evidence that the plasma concentration of IGF1 in females of the C group was higher than that of controls at 28 days (p = 0.0952).

The mean plasma concentrations of leptin are shown in Figure 4.6, p 161. There were no significant differences between supplemented groups and controls in males at either 7 or 28 days (p > 0.1). In females, however, the plasma concentration of leptin was significantly higher in the sunflower (S) group than the control (N) group at both 7 and 28 days (p = 0.0159 in both cases). It was also significantly higher in the canola (C) group at 28 days (p = 0.0317), and there was weak evidence of a similar effect at 7 days (p = 0.0571).

Table 4.7 Plasma concentration of IGF1 by age, sex and feeding group. Values shown are mean plasma concentrations ± SEM for lambs sampled at 7 (± 1) and 28 (± 3) days. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). M is male, F is female. At 7 days, n = 4 for all groups except CM (n = 2), SF (n = 5) and CF (n = 3). At 28 days, n = 4 for male groups and n = 5 for female groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0952; p > 0.1 in all other cases).

Age	Sex	Plasma concentration (ng ml^{-1})		
		N	S	C
7 days	M	150 ± 10	158 ± 9	169 ± 2
	F	126 ± 5	137 ± 16	100 ± 17
28 days	M	319 ± 42	273 ± 9	254 ± 34
	F	186 ± 12 [a]	221 ± 31	228 ± 20 [a]
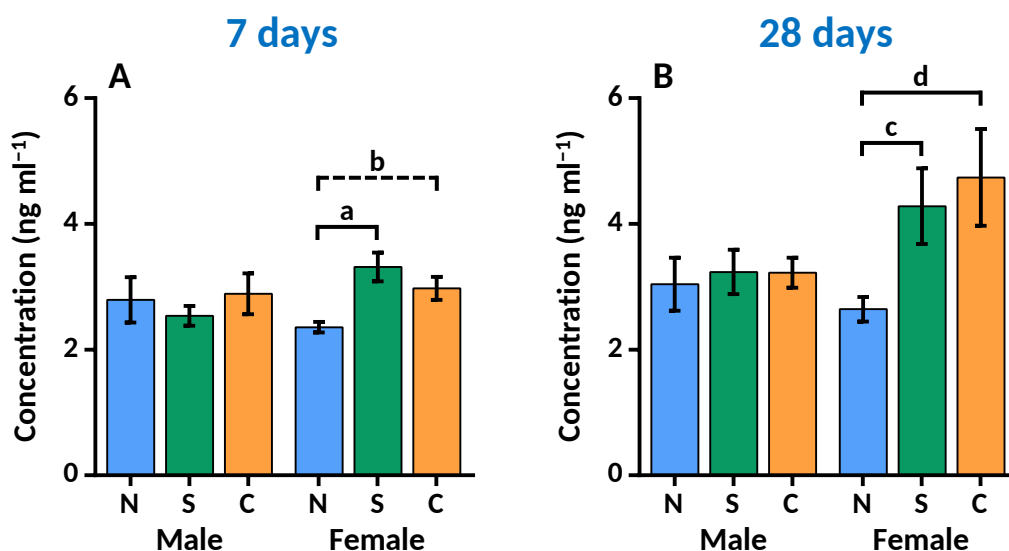
Figure 4.6 Plasma concentration of leptin by age, sex and feeding group. Values shown are mean plasma concentration ± SEM for lambs sampled at 7 (± 1) and 28 (± 3) days. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). At 7 days, n = 4 for all groups except CM (n = 2), SF (n = 5) and CF (n = 3). M is male, F is female. At 28 days, n = 4 and n = 5 for male and female groups respectively. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0159; b: p = 0.0571; c: p = 0.0159; d: p = 0.0317).

4.4.4 Gene expression

Gene expression results are presented in terms of normalised DNA copy number. Results for genes where at least one statistical comparison produced a p-value of less than 0.1 are shown in Figure 4.7, p 162 (adipogenic and metabolic genes; 7 days), Figure 4.8, p 163 (thermogenic genes; 7 days) and Figure 4.9, p 165 (adipogenic, metabolic and thermogenic genes; 28 days). Results for genes where all comparisons produced a p-value of greater than 0.1 are shown in Table 4.8, p 164 (7 days) and Table 4.9, p 166 (28 days).
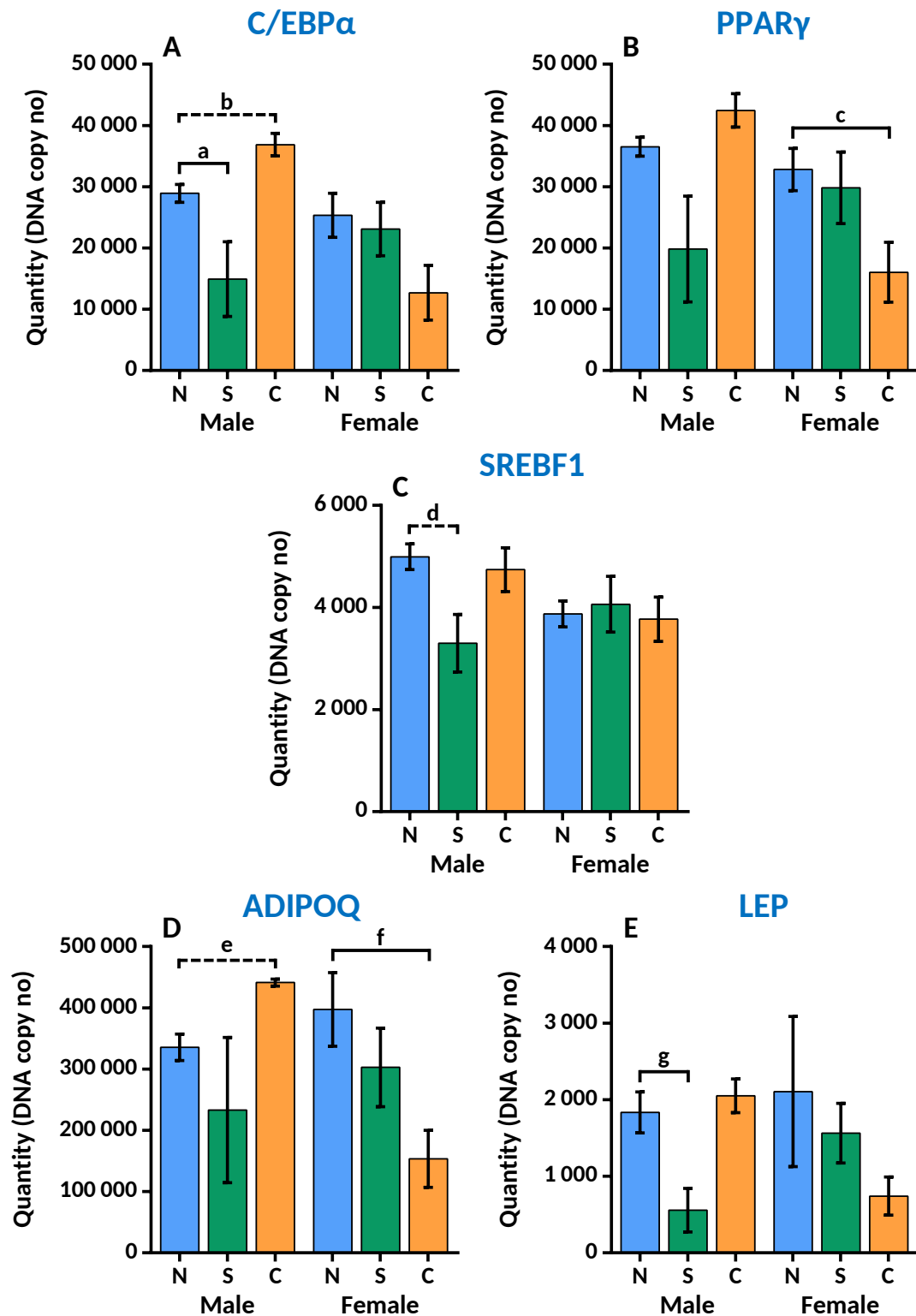
Figure 4.7 Expression of adipogenic and metabolic genes in sternal adipose tissue at 7 days by sex and feeding group. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 7 (± 1) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except C male (n = 3) and S female (n = 5). Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0286; b: p = 0.0571; c: p = 0.0286; d: p = 0.0571; e: p = 0.0571; f: p = 0.0286; g: p = 0.0286; p > 0.1 in all other cases).
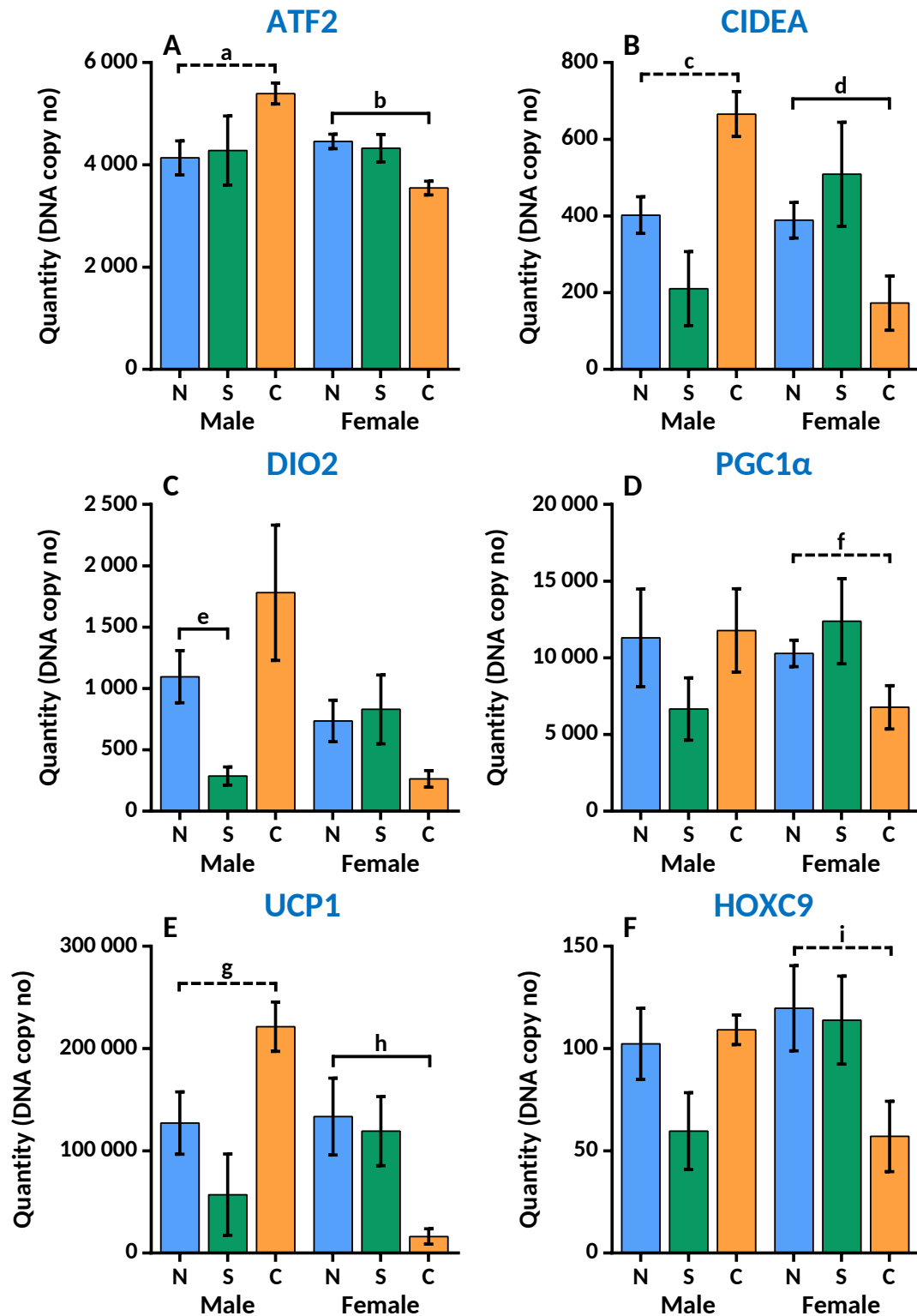
Figure 4.8 Expression of thermogenic and developmental genes in sternal adipose tissue at 7 days by sex and feeding group. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 7 (± 1) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except C male (n = 3) and S female (n = 5). Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0571; b: p = 0.0286; c: p = 0.0571; d: p = 0.0286; e: p = 0.0286; f: p = 0.0571; g: p = 0.0571; h: p = 0.0286; i: p = 0.0571; p > 0.1 in all other cases).

Table 4.8 Gene expression in sternal adipose tissue at 7 days by sex and feeding group. The values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 7 (± 1) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except CM (n = 3) and SF (n = 5); M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (p > 0.1 in all cases).

Category	Gene	Sex	Quantity (DNA copy number)		
			N	S	C
Adipogenesis	NR3C1	M	15 015 ± 844	20 182 ± 2 148	15 669 ± 758
		F	16 729 ± 983	16 735 ± 2 406	18 264 ± 1 772
Metabolism	FABP4	M	1 712 622 ± 115 304	834 442 ± 408 921	2 076 109 ± 379 419
		F	1 755 280 ± 196 260	1 307 602 ± 308 280	838 631 ± 357 009
	GPR120	M	88 ± 27	37 ± 17	86 ± 27
		F	31 ± 5	54 ± 12	38 ± 13
	INSR	M	7 257 ± 1 592	6 012 ± 1 397	8 672 ± 470
		F	7 225 ± 976	6 364 ± 1 032	5 984 ± 616
	RIP140	M	4 264 ± 663	4 924 ± 276	4 655 ± 623
		F	5 179 ± 402	4 549 ± 645	5 452 ± 957
Thermogenesis	PRLR	M	409 ± 73	385 ± 173	350 ± 124
		F	364 ± 71	231 ± 37	368 ± 151
Development	LHX8	M	64 ± 42	16 ± 3	72 ± 53
		F	179 ± 69	109 ± 19	47 ± 22
	PRDM16	M	85 ± 13	80 ± 20	102 ± 18
		F	75 ± 4	86 ± 16	66 ± 10
	SHOX2	M	995 ± 149	1 036 ± 137	1 094 ± 179
		F	988 ± 116	1 196 ± 205	928 ± 68

One male animal in the canola (C) group was a huge outlier for UCP1 expression at 28 days. It had a value of around 132 000 DNA copies, roughly in the middle of the range for 7 day-old animals, but nearly 40 times higher than the next highest figure for 28 day-old animals (around 3 500 copies). It was also an outlier for another thermogenic gene, DIO2, but to a much lesser extent (around 900 copies compared with the next highest figure of around 300 copies), and a minor outlier for the metabolic gene INSR (around 15 000 DNA copies, where the other values were between 3 000 and 10 000 copies). It was suspected initially that an experimental error had been made, but colleagues investigating other adipose tissues (perirenal and pericardial) from the same animals, and who had extracted RNA independently, identified the same outlier for UCP1 (albeit, not to the same extent). This means that any mislabelling could only have occurred at the sampling stage, and it is considered virtually impossible that a 7 day-old lamb could have been confused with a 28
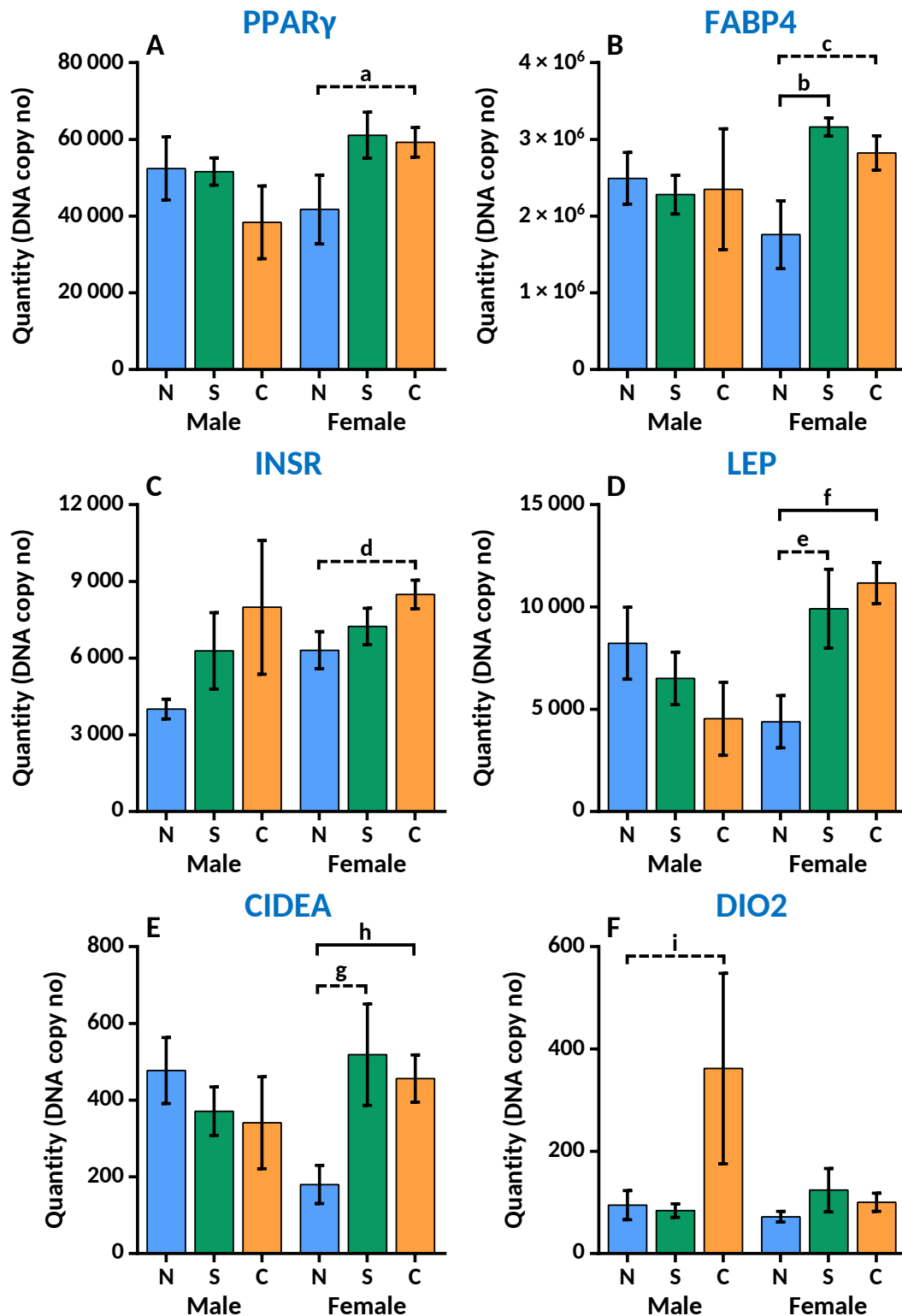
Figure 4.9 Expression of adipogenic, metabolic and thermogenic genes in sternal adipose tissue at 28 days by sex and feeding group. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 28 (± 3) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 and n = 5 for male and female groups respectively. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: $p = 0.0952$; b: $p = 0.0159$; c: $p = 0.0556$; d: $p = 0.0556$; e: $p = 0.0556$; f: $p = 0.0159$; g: $p = 0.0952$; h: $p = 0.0159$; i: $p = 0.0571$; $p > 0.1$ in all other cases).

Table 4.9 Gene expression in sternal adipose tissue at 28 days by sex and feeding group. The values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 28 (± 3) days of age. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 and n = 5 for M and F groups respectively; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (p > 0.1 in all cases).

Category	Gene	Sex	Quantity (DNA copy number)		
			N	S	C
Adipogenesis	C/EBPα	M	14 720 ± 1 384	20 259 ± 2 655	15 685 ± 4 309
		F	11 294 ± 1 599	13 417 ± 2 454	18 075 ± 5 137
	NR3C1	M	26 821 ± 748	24 158 ± 1 038	27 616 ± 4 033
		F	26 145 ± 1 601	25 184 ± 790	24 176 ± 379
	SREBF1	M	3 653 ± 435	4 435 ± 820	3 459 ± 109
		F	3 115 ± 320	3 707 ± 695	4 157 ± 955
Metabolism	ADIPOQ	M	561 094 ± 73 741	587 308 ± 45 809	591 839 ± 173 794
		F	501 666 ± 141 016	727 821 ± 48 311	733 805 ± 60 276
	GPR120	M	904 ± 244	592 ± 72	391 ± 62
		F	287 ± 49	457 ± 92	567 ± 113
	RIP140	M	9 907 ± 684	10 939 ± 826	8 177 ± 734
		F	9 860 ± 528	9 537 ± 1 005	8 868 ± 684
Thermogenesis	ATF2	M	7 531 ± 1 090	6 636 ± 259	6 533 ± 306
		F	6 740 ± 407	7 177 ± 182	6 904 ± 208
	PGC1α	M	1 460 ± 527	1 809 ± 337	3 417 ± 1 466
		F	2 832 ± 1 073	1 747 ± 417	1 793 ± 712
	PRLR	M	113 ± 22	139 ± 24	200 ± 91
		F	94 ± 19	150 ± 35	204 ± 78
	UCP1	M	204 ± 132	559 ± 357	33 137 ± 32 920
		F	159 ± 65	1 142 ± 618	697 ± 372
Development	HOXC9	M	199 ± 46	167 ± 32	123 ± 27
		F	178 ± 37	170 ± 15	213 ± 63
	LHX8	M	268 ± 152	66 ± 45	100 ± 84
		F	154 ± 65	324 ± 160	309 ± 111
	PRDM16	M	56 ± 10	73 ± 12	63 ± 12
		F	69 ± 9	52 ± 11	60 ± 19
	SHOX2	M	1 321 ± 158	1 281 ± 246	1 749 ± 213
		F	1 492 ± 367	1 627 ± 85	1 616 ± 199

day-old animal, not only because of the obvious disparity in size and weight, but also because the ear-tag numbers of all lambs were checked immediately before euthanasia to confirm their identities. Furthermore, the initial RNA concentrations for 7 day-old animals were generally much higher than those of 28 day-old animals (all above 1 000 ng ml^{-1}), and the figure for the outlier (553 ng ml^{-1}) was in the expected range for the latter category. It is believed, therefore, that the animal is a genuine biological outlier, and it has not been removed from the results shown on the preceding pages. However, for completeness, the effect of removing it for the three genes concerned is shown in Table 4.10, below.

A particularly notable feature of the results was the relatively high level of variation in gene expression between animals within each group, at both 7 and 28 days. The exceptions were NR3C1, RIP140 and ATF2, which were quite tightly grouped at both time points. Despite the high level of variation, a specific pattern seemed to emerge across a number of genes of different categories at 7 days of age. Numerically, if not often significantly, the mean gene expression of male lambs was of the form C > N > S, while in females it was of the form N > S > C. This pattern was seen in the adipogenic genes C/EBPα (Figure 4.7A) and PPARγ (Figure 4.7B), the metabolic genes adiponectin (ADIPOQ; Figure 4.7D), FABP4 (Table 4.8), INSR (Table 4.8) and leptin (LEP; Figure 4.7E) and the primary thermogenic gene UCP1 (Figure 4.8E). A similar but modified pattern in which the expression profile in the males was the same, but that in the females of the form S > N > C, was seen in the thermogenic genes CIDEA (Figure 4.8B), DIO2 (Figure 4.8C) and PGC1α (Figure 4.8D). Given the different roles of the genes concerned, these recurring patterns were initially viewed with a certain

Table 4.10 Gene expression of male lambs in sternal adipose tissue at 28 days by feeding group: effect of outlier removal. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 28 (± 3) days of age. The entries in the 'Outlier' column marked '+' include the outlier and those marked '–' exclude it; the outlier was the same animal in all cases. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except the C group after removal of the outlier (n = 3). Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (a: p = 0.0571; p > 0.1 in all other cases).

| Category	Gene	Outlier	Quantity (DNA copy number)		
			N	S	C
Metabolism	INSR	+	4 006 ± 384	6 284 ± 1 498	7 990 ± 2 617
		–	4 006 ± 384	6 284 ± 1 498	5 575 ± 1 430
Thermogenesis	DIO2	+	95 ± 28	84 ± 13 [a]	362 ± 186 [a]
		–	95 ± 28	84 ± 13	176 ± 15
	UCP1	+	204 ± 132	559 ± 357	33 137 ± 32 920
		–	204 ± 132	559 ± 357	217 ± 107

amount of suspicion, with the immediate thought being that this was an artefact arising from differing amounts of cDNA in the 7-day samples. It is mainly for this reason that a considerable amount of time was spent on testing and selecting reference genes (Section 2.4.13, p 105), but despite the concerted attempts to optimise normalisation, the common patterns persisted.

Although statistical testing was initially confined to assessing differences between the S and C groups and controls (N) within each sex, examination of scatter plots revealed yet another curious feature. In the 7-day canola (C) group, expression of a number of genes (mainly those exhibiting the patterns described above) was much higher in males than females, to the extent that there were two discrete groups of values with no overlap. The low number of animals in the male group (n = 3) meant that significance could not be reached under non-parametric testing even with complete separation of the two groups, with a p-value of 0.0571 being achieved in all such cases. Scatter plots by sex and feeding group to illustrate this feature are shown in Figure 4.10, p 169 (adipogenic and metabolic genes) and Figure 4.11, p 170 (thermogenic and developmental genes). As a result of this surprising finding, it was decided to carry out western blotting of the 7-day samples with a UCP1 antibody, with a view to ascertaining whether the apparent disparity between males and females in the C group was a real phenomenon or an anomaly.

At 28 days of age, the recurring patterns of expression and the male/female disparity in the canola (C) group seen at 7 days of age had disappeared. There were fewer significant differences between supplemented groups and controls than at 7 days, and no particular pattern was observed between the expression profiles of different genes or categories, although in females a few genes showed substantially higher (and occasionally significant) expression in the S and C groups than in the control group (N). This profile was seen in the adipogenic gene PPARγ (Figure 4.9A), the metabolic genes FABP4 (Figure 4.9B), adiponectin (ADIPOQ; Table 4.9) and leptin (LEP; Figure 4.9D), and the thermogenic genes CIDEA (Figure 4.9E) and UCP1 (Table 4.9). Of these, however, only FABP4 for the S group and leptin and CIDEA for the C group were significant (p = 0.0159 in all cases). There were no significant differences in the males between the S and C groups and controls (N).
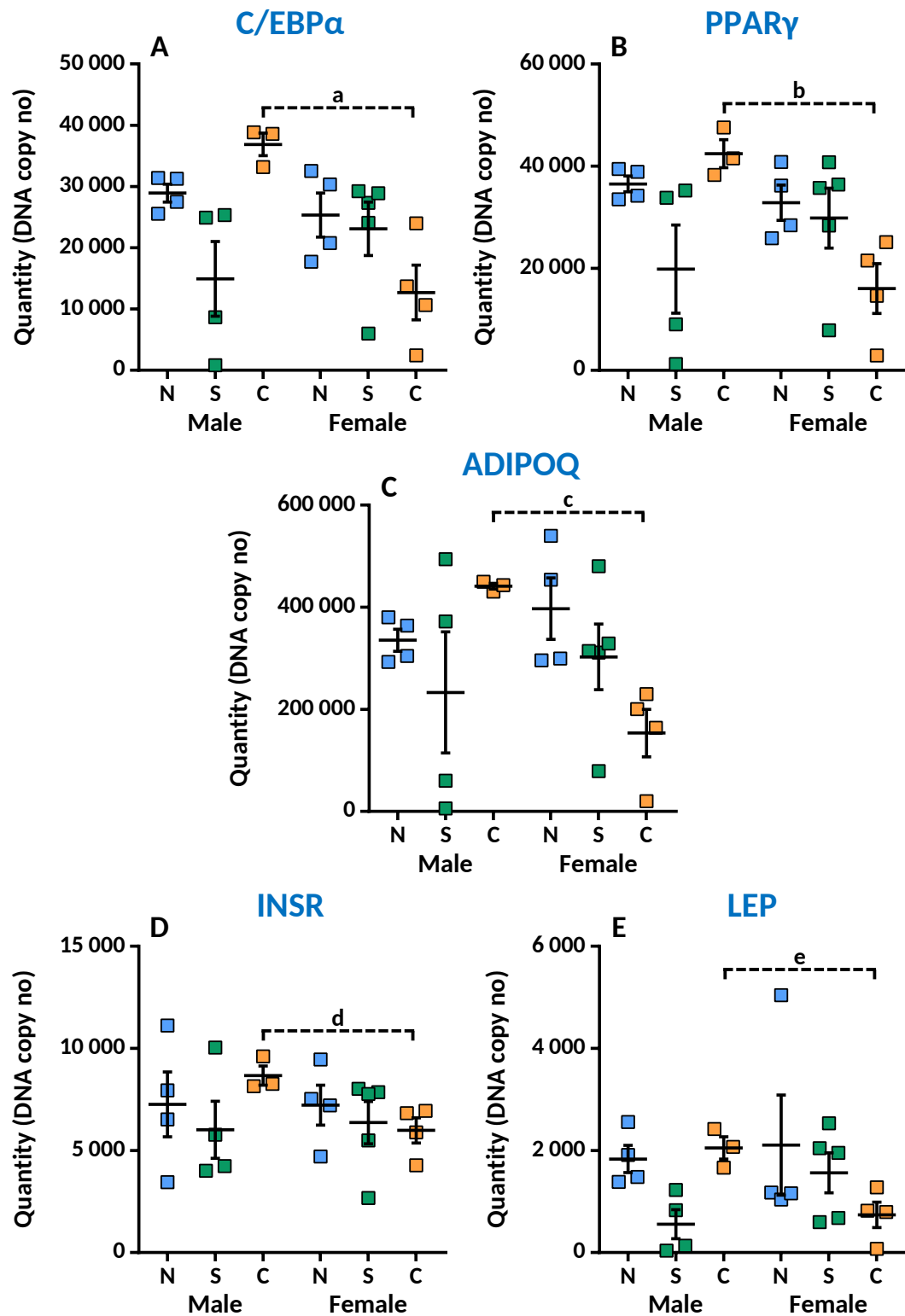
Figure 4.10 Scatter plot of adipogenic and metabolic gene expression in sternal adipose tissue at 7 days by sex and feeding group. Values shown are normalised DNA copy number from lambs sampled at 7 (± 1) days of age. Horizontal lines and error bars represent the mean ± SEM. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except C male (n = 3) and S female (n = 5). Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. Males and females in the C group were compared by Mann-Whitney U-tests (a–e: p = 0.0571).
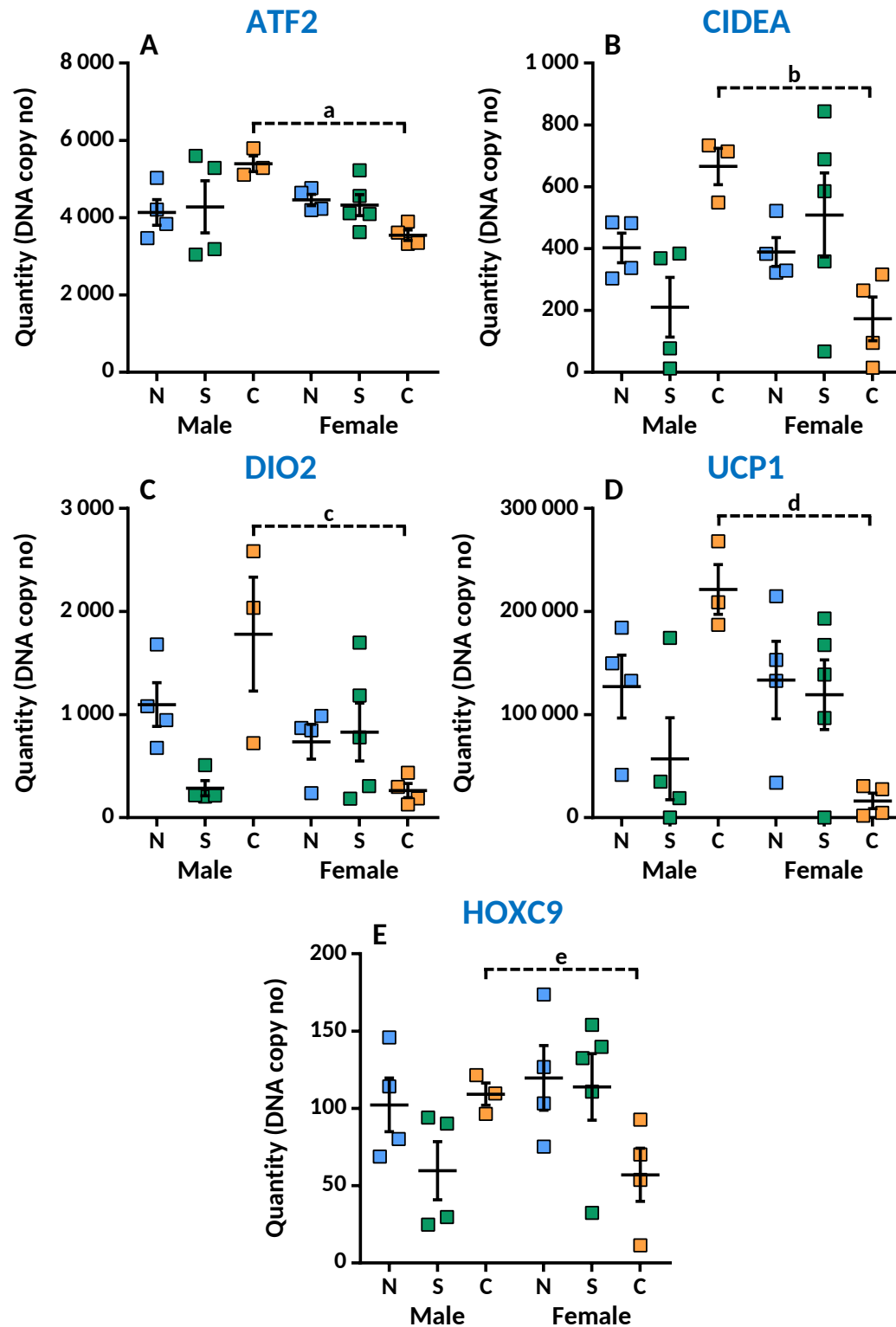
Figure 4.11 Scatter plot of thermogenic and developmental gene expression in sternal adipose tissue at 7 days by sex and feeding group. Values shown are normalised DNA copy number from lambs sampled at 7 ($\pm$ 1) days of age. Horizontal lines and error bars represent the mean $\pm$ SEM. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except C male (n = 3) and S female (n = 5). Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, KDM2B, RPLP0 and TBP. Males and females in the C group were compared by Mann-Whitney U-tests (a–e: p = 0.0571).

4.4.5 Protein expression

SDS-PAGE and western blotting with UCP1 antibody was carried out with the 7 day-old samples. Staining of membranes with Ponceau S dye indicated that protein loading was consistent across the samples. An example image of a western blot membrane stained with Ponceau S is shown in Figure 4.12, below, and the results of a densitometric analysis of the most prominent dye-stained band are shown in Table 4.11, below.
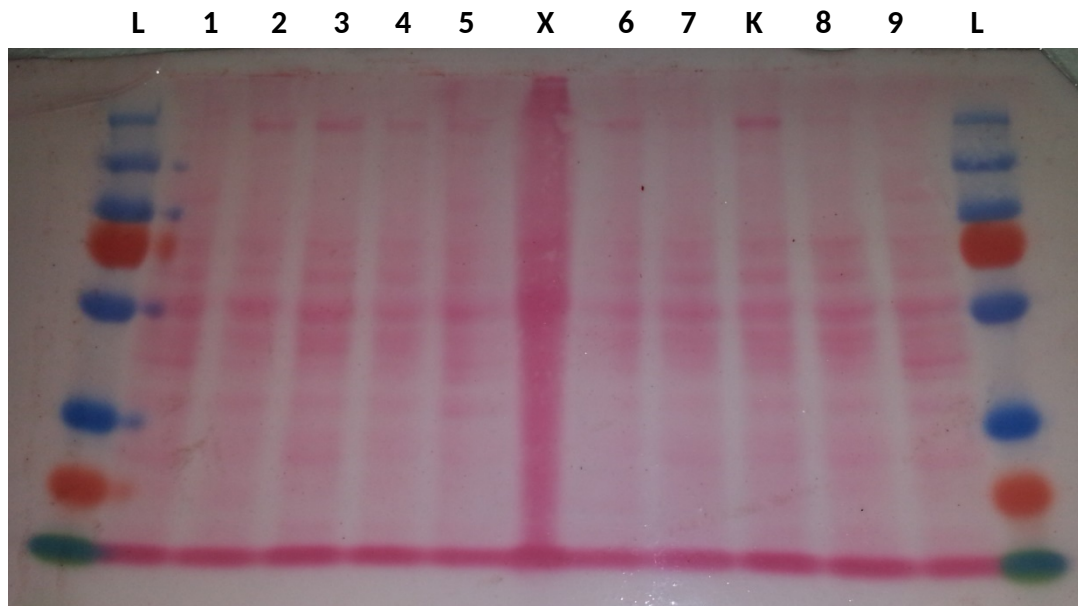
Figure 4.12 Example image of a western blot membrane stained with Ponceau S dye for 7-day sternal adipose tissue. The dye was used to check the consistency of protein loading in poly-acrylamide gels. The letters above the lanes indicate protein ladders (L), negative control for UCP1 (X), calibrator (K) or adipose tissue samples (1–9). The negative control was a different tissue, liver, which explains the different banding pattern in that lane.

Table 4.11 Relative total protein loading of polyacrylamide gels for 7-day sternal adipose tissue by sex and feeding group. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except C male (n = 3) and S female (n = 5). M is male, F is female. Gels were western blotted onto membranes, then stained with Ponceau S dye. Staining of the bottom (most prominent) protein band was analysed by densitometry and expressed relative to the NM group in arbitrary units (AU). All groups were compared by Kruskal-Wallis test, and there were no significant differences (p > 0.1).

	Relative expression (AU)		
Sex	N	S	C
M	1.00 ± 0.05	1.06 ± 0.07	1.11 ± 0.02
F	1.10 ± 0.05	1.05 ± 0.04	1.03 ± 0.03

An example image of a western blot membrane treated with UCP1 antibody is shown in Figure 4.13, below. There is one prominent band on the membrane which corresponds with the expected molecular weight for UCP1 (around 33 kDa). UCP1 protein expression by sex and feeding group is shown in Figure 4.14, p 173. The results are shown both as a bar chart and a scatter plot, to enable comparison with the UCP1 gene expression analyses in Figure 4.8E, p 163, and Figure 4.11D, p 170. There were no significant differences between the S or C groups and controls (N) in either the males or females ($p > 0.1$), and the distinct separation of male and female samples in the C group observed in the UCP1 gene analysis (Figure 4.11D, p 170) was not replicated.

The relationship between UCP1 relative gene and protein expression is shown in Figure 4.15, p 173. UCP1 gene and protein expression was positively correlated overall (Spearman correlation coefficient $\rho = 0.6052$, $p = 0.0017$), though there was considerable variation. One sample in particular, a female in the C group, had very high relative expression of UCP1 protein (0.75), but very low relative expression of UCP1 mRNA (0.01), and is an obvious outlier on the protein axis in Figure 4.15, p 173, where it has been ringed in red. If this animal is removed from the UCP1 protein analysis, distinct separation between males and females in the C group is restored (Figure 4.14B, p 173), though to a much lesser extent than was observed in the UCP1 gene analysis (Figure 4.11D, p 170).
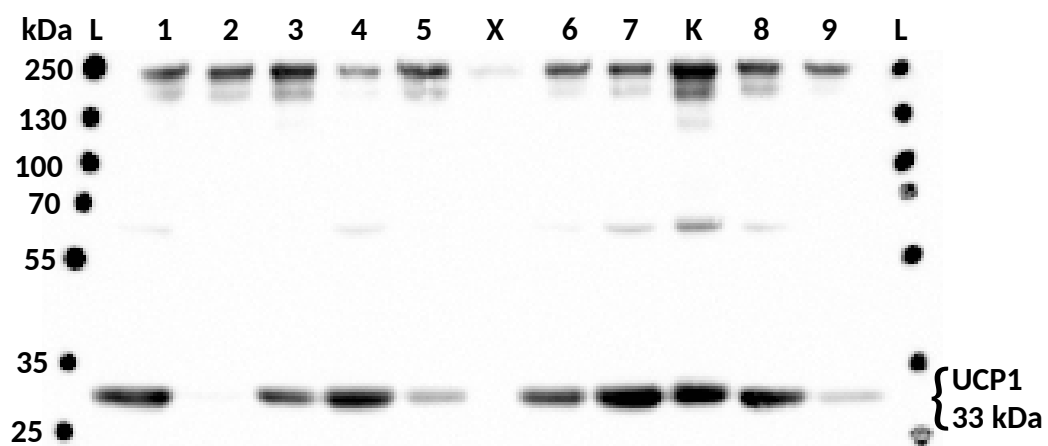
Figure 4.13 Example image of a western blot membrane treated with UCP1 antibody for 7-day sternal adipose tissue. The letters above the lanes indicate protein ladders (L), negative control for UCP1 (X), calibrator (K) or adipose tissue samples (1–9). The negative control is liver, which does not express UCP1.
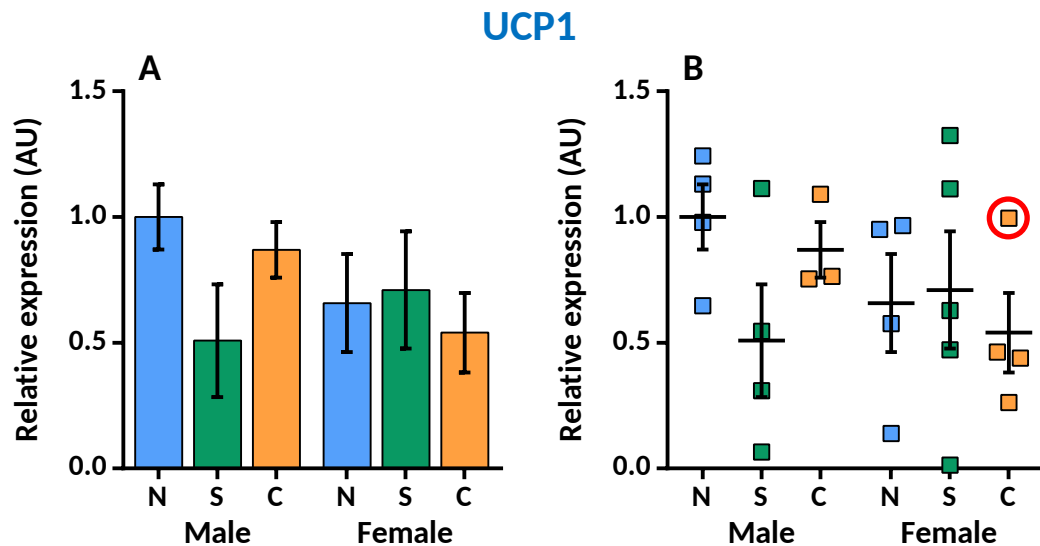
Figure 4.14 UCP1 protein expression in sternal adipose tissue at 7 days by sex and feeding group. Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C); n = 4 for all groups except C male (n = 3) and S female (n = 5). Values shown in the bar chart (**A**) are the mean expression in arbitrary units (AU) relative to male controls (N) ± SEM of protein from lambs sampled at 7 (± 1) days of age. Values in the scatter plot (**B**) are expression in AU of individual samples relative to the average of the male control group (N). The point ringed in red corresponds to an outlier in the plot of relative mRNA against relative protein expression (Figure 4.15, below). S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (p > 0.1 in all cases). Males and females in the C group were also compared by Mann-Whitney U-test (p > 0.1).
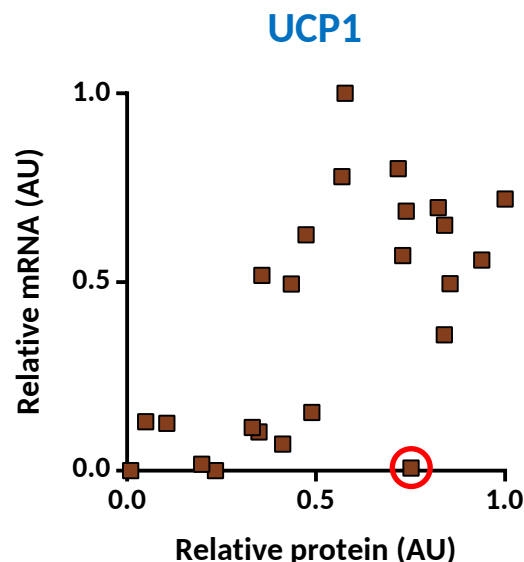
Figure 4.15 Relationship between UCP1 relative gene and protein expression in 7-day sternal adipose tissue. Relative UCP1 mRNA expression in arbitrary units (AU) is shown plotted against relative UCP1 protein expression in AU for individual lambs sampled at 7 (± 1) days of age (n = 24). The point ringed in red is an outlier that is highlighted in the plot of UCP1 protein expression by sex and feeding group (Figure 4.14, above). A Spearman correlation was performed, and it was found that there is a positive correlation between UCP1 mRNA and protein expression (Spearman's rank correlation coefficient ρ = 0.6052, p = 0.0017).

4.4.6 Histology and immunohistochemistry

Tissue sections from all the 7 day-old samples were stained with H&E. Three sections, one from a female in the control (N) group and two from males in the canola (C) group, did not produce useable images, either because the section was sliced too thick, and therefore contained too many layers of cells, or because it only contained non-adipose (eg connective) tissue. The failure of the two canola male samples was particularly disappointing, as the canola group was already short on numbers, and it was the group of greatest interest. Unfortunately, however, lack of time prevented repetition of the flawed sections. Images of 0.5 mm-square sections of tissue stained with H&E are shown in Figure 4.16, p 175 (control males), Figure 4.17, p 176 (control females), Figure 4.18, p 177 (sunflower males), Figure 4.19, p 178 (sunflower females) and Figure 4.20, p 179 (canola males and females). As all the images are of the same-sized area of tissue and at the same magnification, they can be directly compared. The relative (UCP1) protein expression (RPE; maximum 1.00) obtained from western blotting has been shown above each image, so that any relationship between protein expression and tissue morphology can be qualitatively assessed.

In general, the tissue morphology can be classified into two main categories: (1) tissue with a stereotypical WAT morphology, essentially consisting of compacted unilocular cells, albeit with a few much smaller multilocular cells between them; and (2) tissue with areas of continuous, if less compacted, unilocular cells interspersed with well-defined patches of smaller multilocular cells that resemble BAT. Examples of the former are Figure 4.16A, p 175, Figure 4.19A, p 178, and Figure 4.20C, p 179; examples of the latter are Figure 4.17C, p 176, Figure 4.19C, p 178, and Figure 4.20B, p 179. One sample had tissue of both categories, with certain regions being 'patchy' and other regions being more WAT-like (Figure 4.18C and Figure 4.18D, p 177). Another exception to the general pattern was a sample from a female in the sunflower (S) group (Figure 4.19B, p 178) which was closer to stereotypical BAT. It consisted primarily of small multilocular cells, with larger clustered or isolated unilocular cells scattered throughout. This was also the tissue with the highest RPE (1.00).

Tissue sections from 12 of the 7-day samples (all 8 canola (C) samples plus 2 each (1M, 1F) from the control (N) and sunflower (S) groups) were also subjected to IHC processing and treatment with a UCP1 antibody, with a view to confirming that UCP1 was primarily expressed in areas of tissue identified as BAT. As expected, the brown staining from the UCP1 antibody was observed to be concentrated around the small multilocular cells that are indicative of BAT. Example images of 0.5 mm-square sections of tissue processed by IHC are shown in Figure 4.21, p 180, and Figure 4.22, p 181.
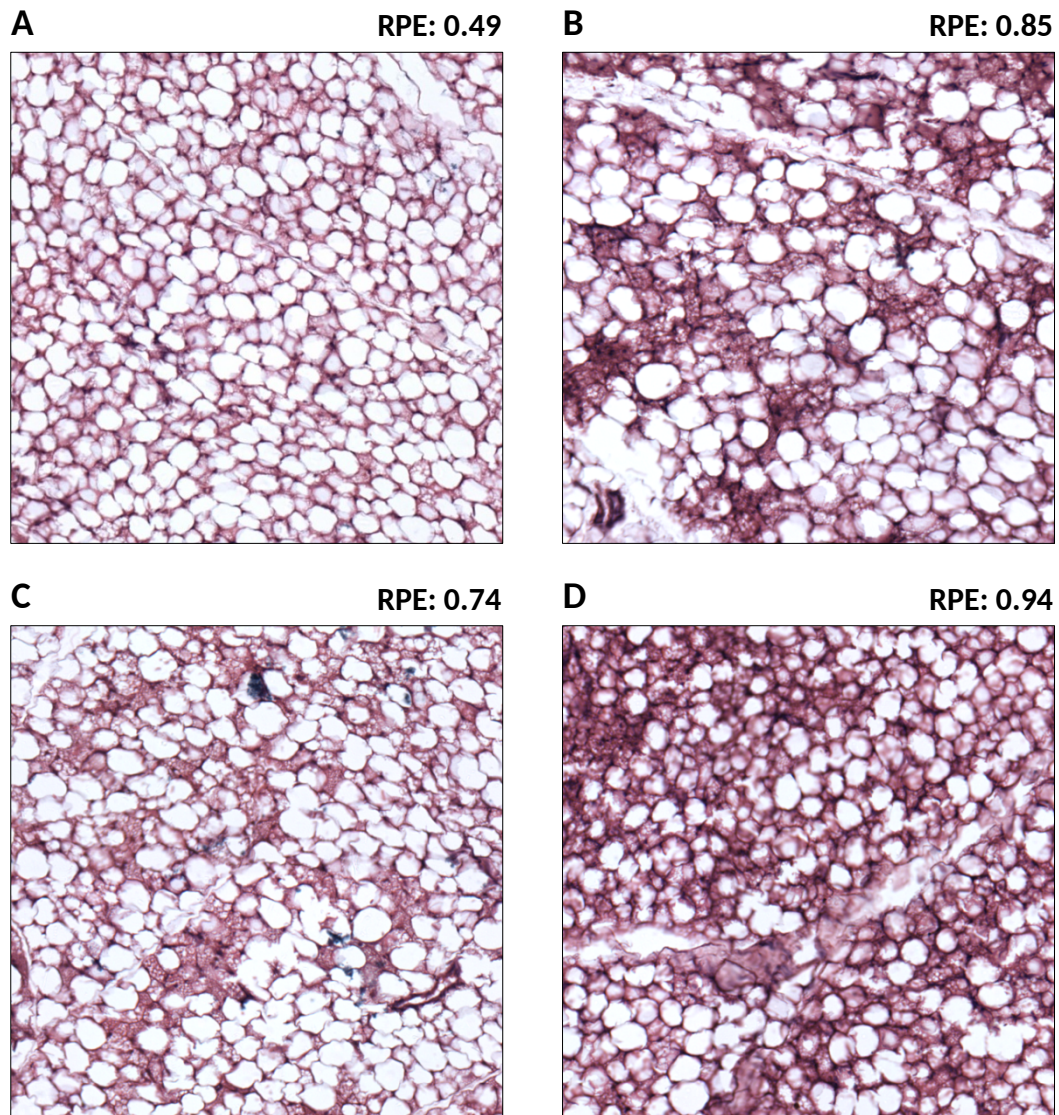
Figure 4.16 H&E-stained sections of 7-day sternal adipose tissue of male lambs in the control (N) feeding group. H&E is haematoxylin and eosin. Lambs were sampled at 7 (± 1) days of age. All images are from different animals. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).

There were no obvious morphological features that distinguished the different feeding groups or sexes, and the relationship between tissue morphology and RPE was inconsistent. At the extremes, the most BAT-like tissue had the highest RPE, as already noted, and the tissue with the lowest RPE (0.01)[34] had the most WAT-like morphology, with very few multilocular cells between the large unilocular cells (Figure 4.19D, p 178). Between the two extremes there was more variation. Figure 4.16, above, shows a good progression for the control males, with tissue morphology changing from very WAT-like to increasingly

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[34] This tissue also had the second lowest relative mRNA expression of the 7 day-old animals.
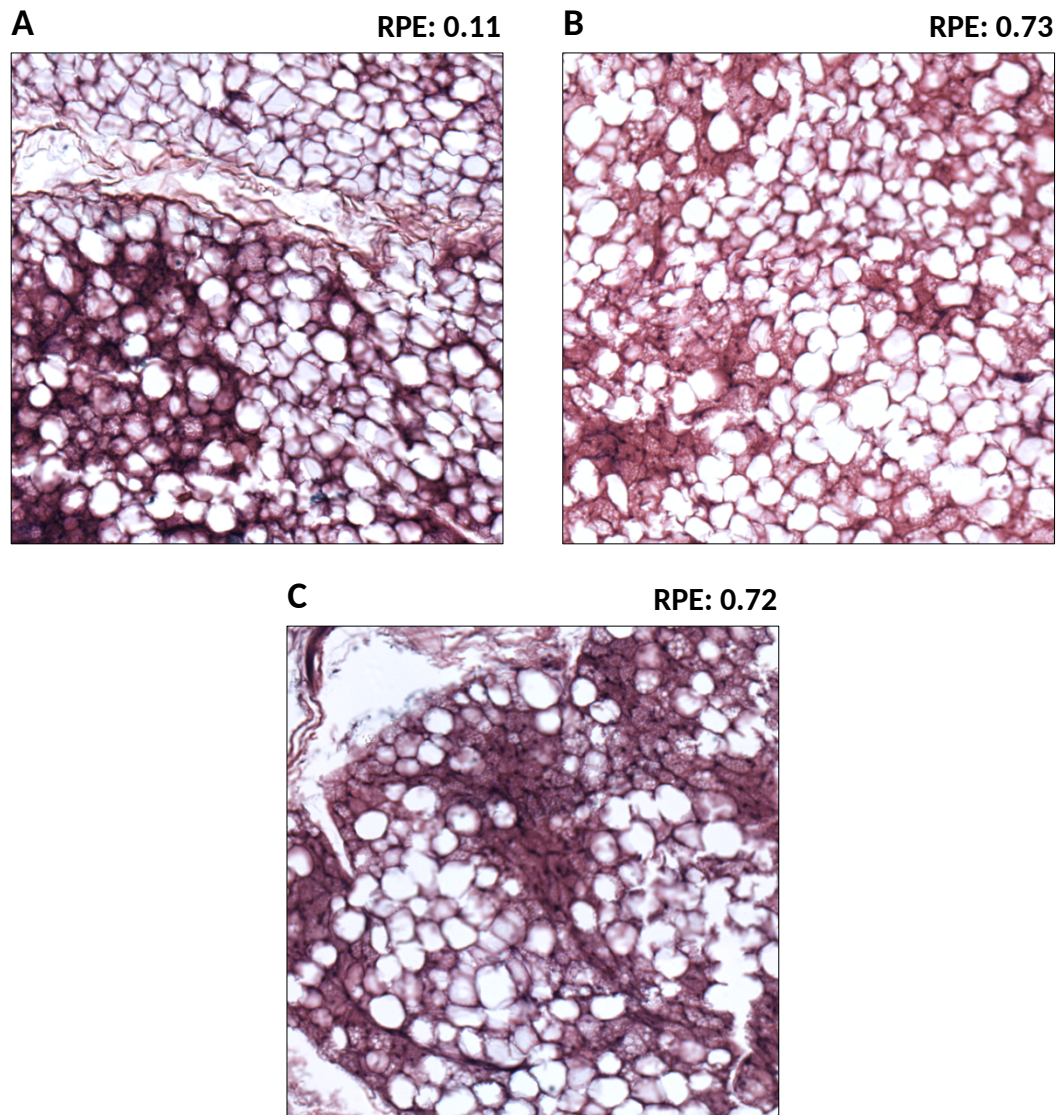
Figure 4.17 H&E-stained sections of 7-day sternal adipose tissue of female lambs in the control (N) feeding group. H&E is haematoxylin and eosin. Lambs were sampled at 7 (± 1) days of age. All images are from different animals. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).

more 'patchy' as the RPE increases. There is also a reasonable progression in the sunflower female group (Figure 4.19, p 178), which contains both the extremes of RPE. In the other groups, however, there are clear inconsistencies. For example, the tissue morphologies of the control females in Figure 4.17A and Figure 4.17B, above, are similar, but the respective RPE values are 0.11 and 0.73. Further inconsistencies occur across groups. For example, the WAT-like tissues in Figure 4.16A, p 175, and Figure 4.20A, p 179, have RPEs of 0.49 and 0.58 respectively, whereas the 'patchy' tissues in Figure 4.18A, p 177, and Figure 4.20B, p 179, have respective RPEs of 0.23 and 0.20.
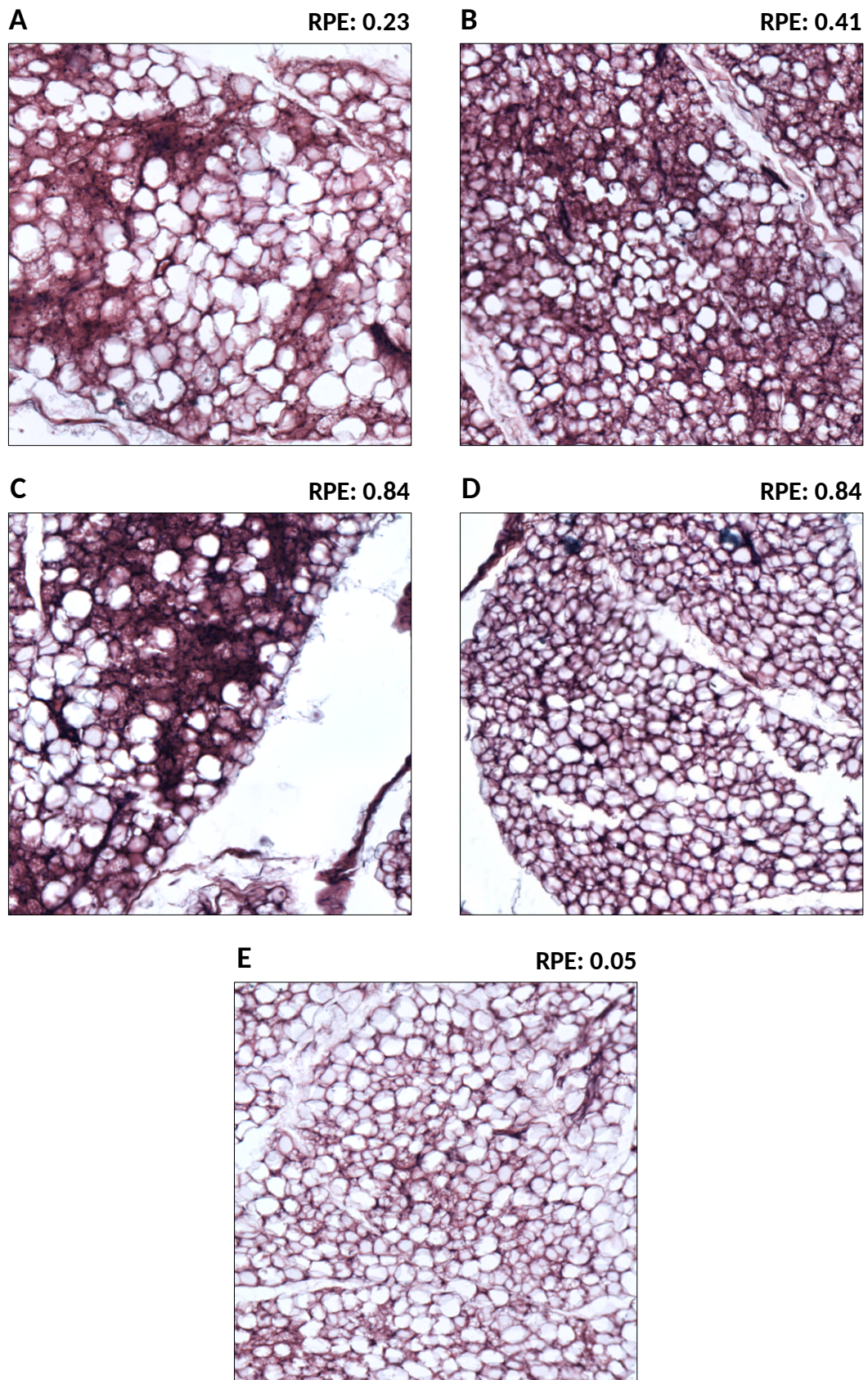
Figure 4.18 H&E-stained sections of 7-day sternal adipose tissue of male lambs in the sunflower (S) feeding group. H&E is haematoxylin and eosin. Lambs were sampled at 7 (± 1) days of age. Images **C** and **D** are from the same animal, all other images are from different animals. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).
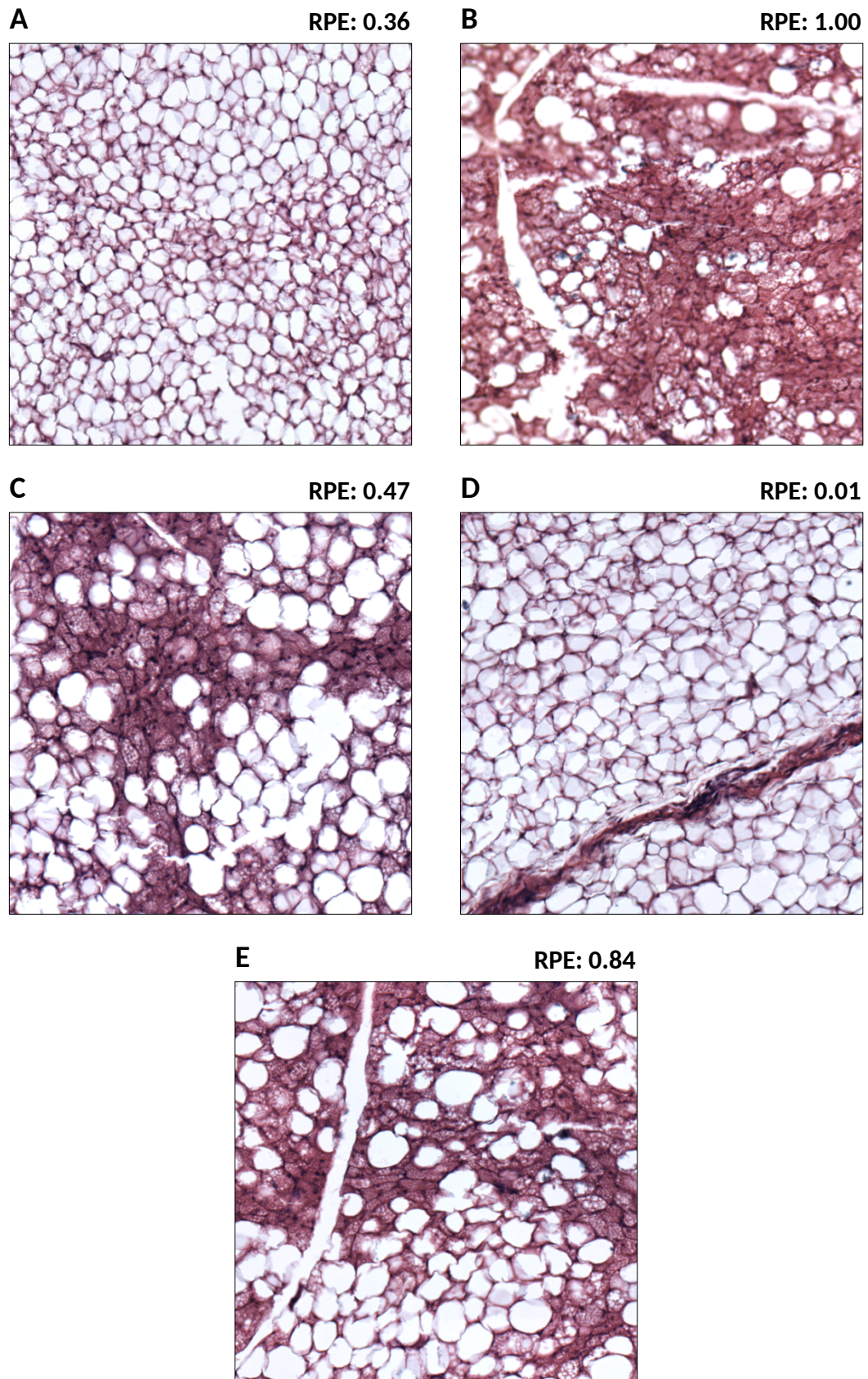
Figure 4.19 H&E-stained sections of 7-day sternal adipose tissue of female lambs in the sunflower (S) feeding group. H&E is haematoxylin and eosin. Lambs were sampled at 7 (± 1) days of age. All images are from different animals. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).
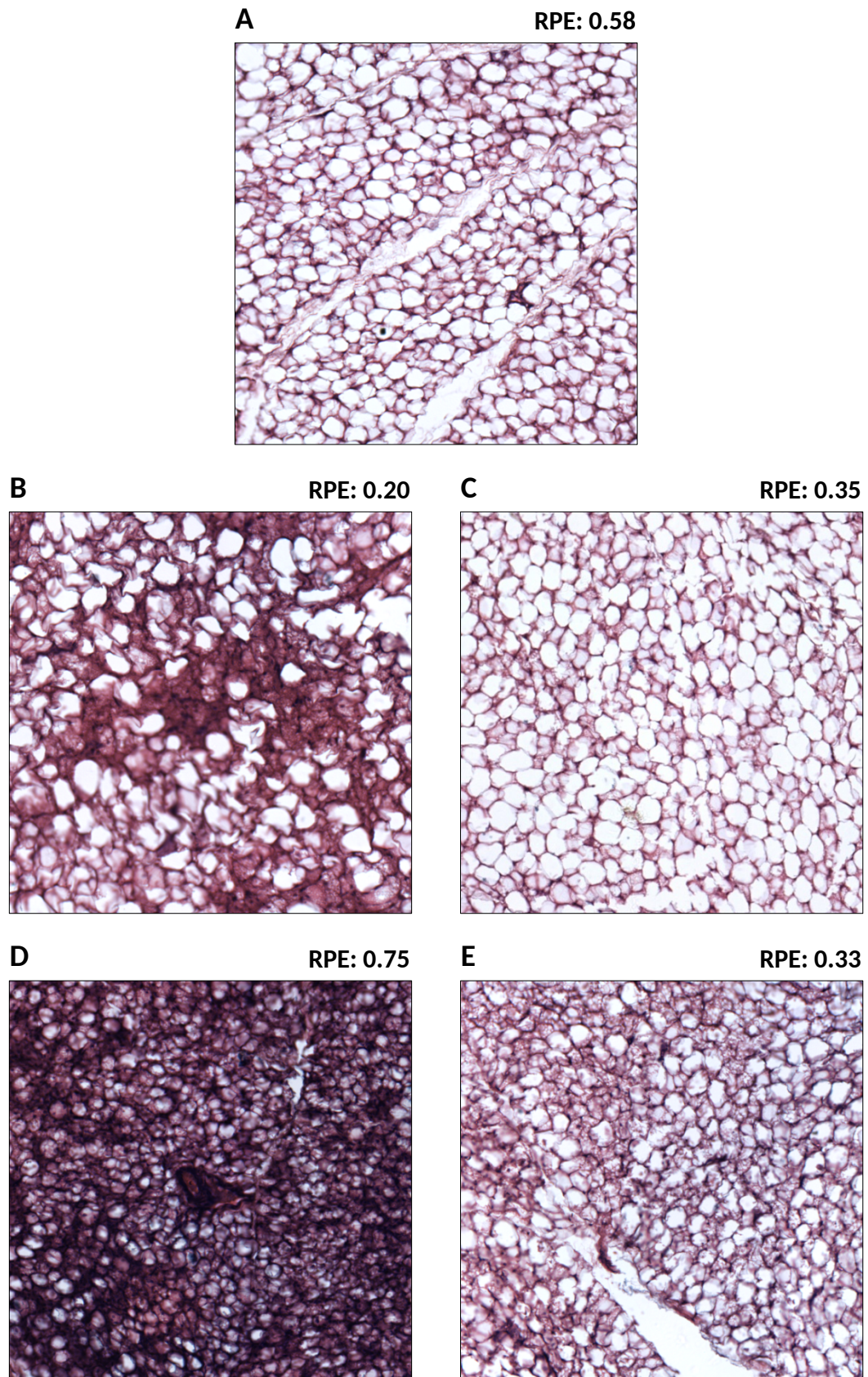
Figure 4.20 H&E-stained sections of 7-day sternal adipose tissue of male and female lambs in the canola (C) feeding group. H&E is haematoxylin and eosin. Lambs were sampled at 7 (± 1) days of age. All images are from different animals, **A** is male; **B**, **C**, **D** and **E** are females. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).

Control female (NF)

A

UCP1-negative

B   RPE: 0.73

UCP1-positive

Sunflower female (SF)

C

UCP1-negative

D   RPE: 0.47

UCP1-positive
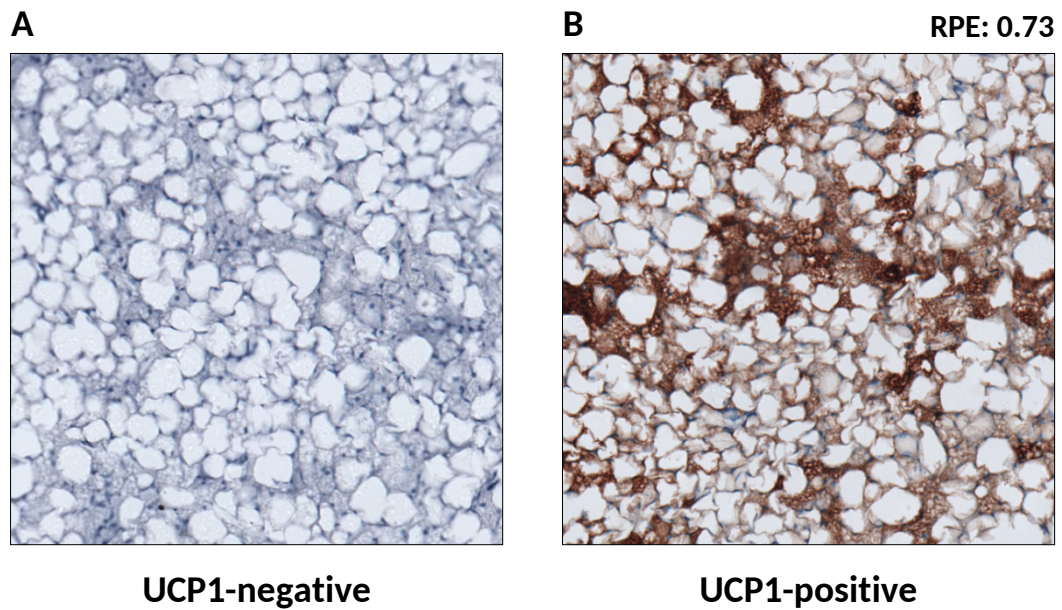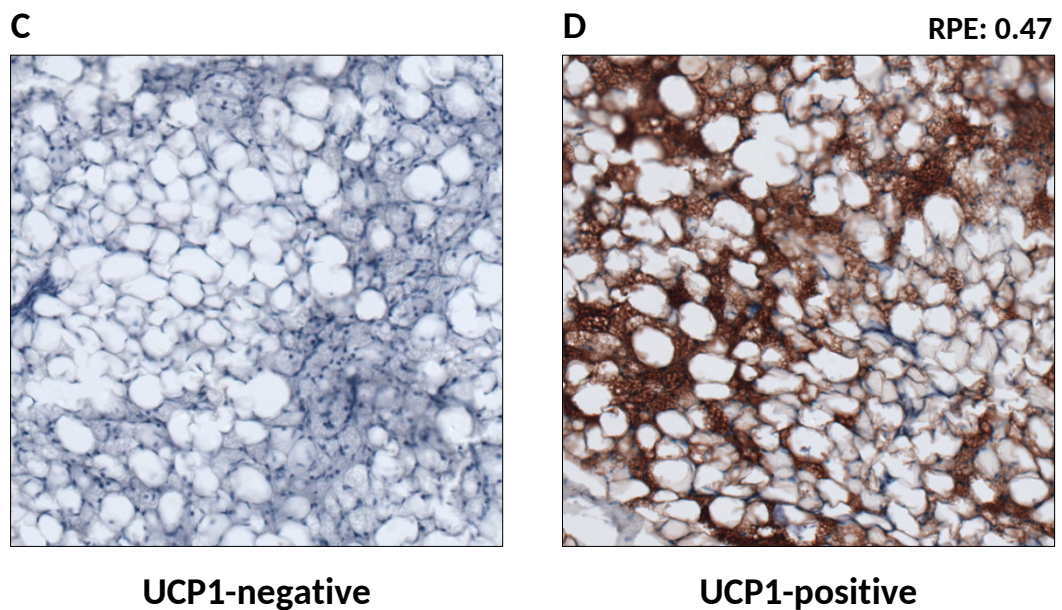
Figure 4.21 Example sections of 7-day sternal adipose tissue of lambs in the control (N) and sunflower (S) feeding groups treated with UCP1 antibody. The counterstain is haematoxylin. Lambs were sampled at 7 (± 1) days of age. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. Images of the corresponding haematoxylin and eosin stains for images **A/B** and **C/D** respectively are shown in Figure 4.17B, p 176, and Figure 4.19C, p 178. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).

Canola female (CF)

A

B   RPE: 0.75

UCP1-negative   **UCP1-positive**

Canola female (CF)

C

D   RPE: 0.33

UCP1-negative   **UCP1-positive**
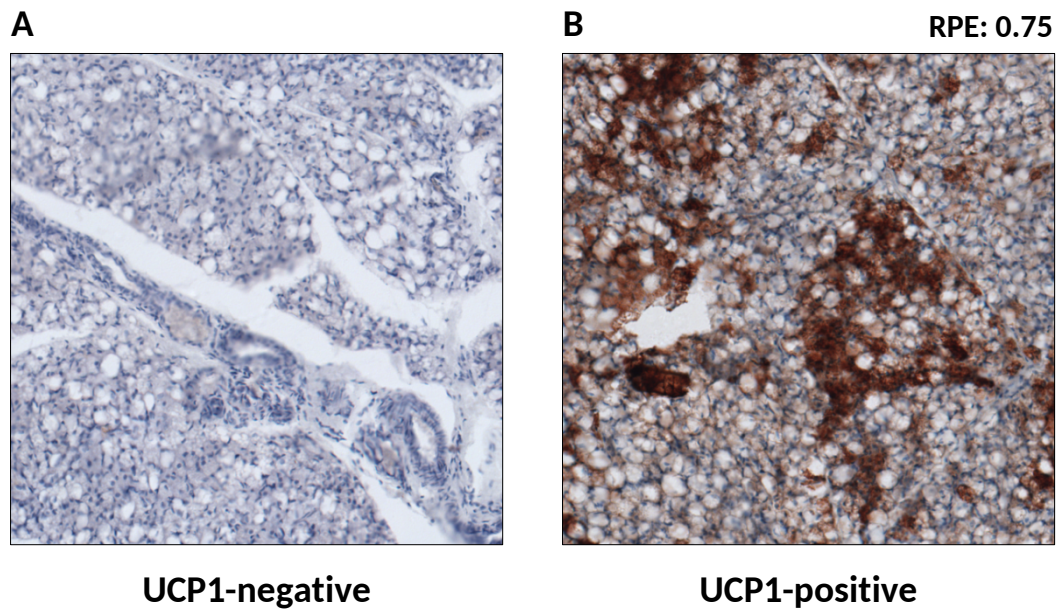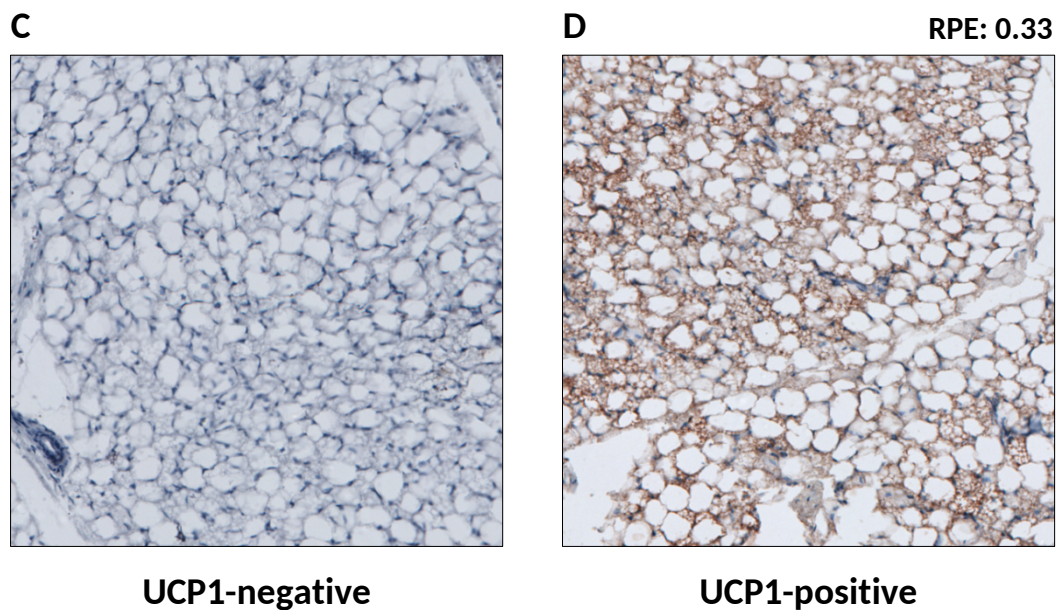
Figure 4.22 Example sections of 7-day sternal adipose tissue of lambs in the canola (C) feeding group treated with UCP1 antibody. Images **A/B** and **C/D** are different animals. The counterstain is haematoxylin. Lambs were sampled at 7 (± 1) days of age. Each image is of a 0.5 mm square of tissue, photographed at an original magnification of 20X. Images of the corresponding haematoxylin and eosin stains for images **A/B** and **C/D** respectively are shown in Figure 4.20D and Figure 4.20E, p 179. RPE is relative (UCP1) protein expression from western blotting (maximum 1.00).

A further observation concerns the canola female outlier discussed in Section 4.4.5, p 171, which had very high protein expression (RPE 0.75) but minimal mRNA expression. The image of H&E-stained tissue from this animal is poor (Figure 4.20D, p 179), as the section was cut too thick and contains multiple layers of cells. It is therefore difficult to interpret, and would have been excluded were it not of particular interest. However, large clusters of nuclei close together are suggestive of 'patchy' tissue, and the negative control from IHC (Figure 4.22A, p 181) also shows a 'patchy' morphology with large numbers of nuclei. This is confirmed by treatment with the UCP1 antibody, which produced well-defined areas of dark brown staining (Figure 4.22B, p 181). It appears, therefore, that the high level of protein expression in this sample that was detected by western blotting is supported by the tissue morphology and IHC.

A final observation concerns a male in the canola group. This animal had the highest relative UCP1 mRNA expression (Figure 4.11D, p 170), but a much lower RPE (0.58). The tissue section was homogenous and of clear WAT-like morphology (Figure 4.20A, p 179), with very few multilocular cells visible in the image. Although this sample was one of those selected for IHC, the UCP1-positive tissue separated from its slide during processing, and could not therefore be used. Although it would have been interesting to see the UCP1 expression pattern in this tissue, there was insufficient time to repeat the procedure.

4.4.7 Statistical power

The results of statistical power calculations for tests on tissue weights, plasma metabolites, gene expression and UCP1 protein expression are shown in Table 4.12, p 183. As can be seen from the table, for most tests the study was considerably underpowered. Sample sizes of 20 for each age/feeding group/sex combination would have been the minimum number required to achieve a power of 80 % with an effect size of 1.0 (Section 4.4.7, p 182).

4.4.8 Summary of results

A summary of the results for this chapter is shown in Table 4.13, p 184.

Table 4.12 Estimates of statistical power. Results of *post hoc* statistical power calculations for two-tailed Mann-Whitney U-tests comparing the sunflower (S) or canola (C) groups with controls. In determining effect sizes for tissue weights, plasma metabolites and gene expression, the absolute percentage differences between the means of each supplemented group (S or C) and the control group, and the coefficients of variation for each group, were averaged across all tissues, metabolites or genes.

Item	7 days				28 days			
	Male		Female		Male		Female	
	S	C	S	C	S	C	S	C
	%	%	%	%	%	%	%	%
Relative tissue weights	8	10	8	5	8	6	70	38
Plasma metabolites	8	7	35	34	8	9	34	49
Gene expression	20	10	9	25	11	14	22	26
UCP1 protein expression	30	9	5	6				

4.5 Discussion

4.5.1 Body and tissue weights

4.5.1.1 A sunflower oil supplement given to lactating mothers may increase the body weight of their female suckling lambs, but the results are inconclusive

It was impossible to allocate lambs to feeding groups such that mean birth weights were identical. When lambs were born, their mothers were allocated immediately to one of the three groups, with the principal objectives being to ensure that the numbers of lambs in each feeding group, both in total and by sex, were similar. Likewise, when the time came for one lamb of a twin pair to be sampled, selection was based on the need to ensure a balance of sex by feeding group and sampling age. This was effectively a 'moving target', as some lambs were being sampled before others had even been born. There is therefore some variation between the mean birth weights by feeding group, sex and sampling age, even though the differences are not significant.[35] Furthermore, there was some variation (± 1–3 days) in the age of the lambs in each of the two age-based sampling groups. To confound these two variables, therefore, body weight was presented in terms of the gain in body weight per day per kg birth weight. On that basis, the weight gain of females in the sunflower group was significantly higher than those in the control group at 7 days of age, with weak evidence for the same effect at 28 days of age. However, there was no significant

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[35] Strictly speaking a statistical analysis of birth weight is probably not appropriate, as mean birth weight is a function of allocation rather than experimental outcome.

Table 4.13 Summary of results. An up arrow (↑⇑) indicates that body weight gain, tissue weight, temperature, concentration or expression is higher in the sunflower (S) or canola (C) group than in controls, a down arrow (↓⇓) indicates that measurements are lower in the S or C groups than in controls, and a horizontal dash (–) indicates that there is no difference between the S or C groups and controls. An outline arrow (⇑⇓) indicates weak evidence of a change (ie $0.05 \leq p < 0.1$). Gene and protein expression were tested in sternal adipose tissue. M is male, F is female.

Item	Category	Gene/tissue	7 days				28 days			
			M		F		M		F	
			S	C	S	C	S	C	S	C
Body weight gain	Relative		–	–	↑	–	–	–	⇑	–
Adipose tissue weight	Absolute	Omental	–	–	–	–	–	–	↑	⇑
		Pericardial	–	–	–	–	–	–	–	–
		Perirenal	–	–	–	–	–	–	↑	–
		Sternal	–	–	↑	–	–	–	⇑	↑
		Combined	–	–	–	–	–	–	↑	↑
	Relative	Omental	–	–	–	–	–	–	↑	–
		Pericardial	–	–	–	–	–	–	⇑	–
		Perirenal	–	–	–	–	–	–	↑	–
		Sternal	–	–	–	–	–	–	⇑	⇑
		Combined	–	–	–	–	–	–	↑	–
Rectal temperature			↓	–	–	–	–	–	–	–
Plasma concentration	Metabolic	IGF1	–	–	–	–	–	–	–	⇑
		Leptin	–	–	↑	⇑	–	–	↑	↑
Gene expression	Adipogenesis	C/EBPα	↓	⇑	–	–	–	–	–	–
		NR3C1	–	–	–	–	–	–	–	–
		PPARγ	–	–	–	↓	–	–	–	⇑
		SREBF1	⇓	–	–	–	–	–	–	–
	Metabolism	ADIPOQ	–	⇑	–	↓	–	–	–	–
		FABP4	–	–	–	–	–	–	↑	⇑
		GPR120	–	–	–	–	–	–	–	–
		INSR	–	–	–	–	–	–	–	⇑
		LEP	↓	–	–	–	–	–	⇑	↑
		RIP140	–	–	–	–	–	–	–	–
	Thermogenesis	ATF2	–	⇑	–	↓	–	–	–	–
		CIDEA	–	⇑	–	↓	–	–	⇑	↑
		DIO2	↓	–	–	–	–	⇑	–	–
		PGC1α	–	–	–	⇓	–	–	–	–
		PRLR	–	–	–	–	–	–	–	–
		UCP1	–	⇑	–	↓	–	–	–	–
	Development	HOXC9	–	–	–	⇓	–	–	–	–
		LHX8	–	–	–	–	–	–	–	–
		PRDM16	–	–	–	–	–	–	–	–
		SHOX2	–	–	–	–	–	–	–	–
Protein expression	Thermogenesis	UCP1	–	–	–	–				

difference between the mean body weights of supplemented groups and controls at 7 and 28 days of age for either sex. It is therefore impossible to definitively conclude that the higher relative weight gain in females is attributable to the sunflower oil supplement, as it could simply be a function of normal 'catch up' growth by (relatively) low birth weight individuals.

4.5.1.2 A sunflower oil supplement given to ewes during lactation increases the relative adipose tissue weight of their 28 day-old female suckling lambs

The hypothesis that fat supplements given to lactating ewes would increase the adipose tissue mass of their suckling lambs is partially supported, but the effect was only seen in female lambs, and only with a sunflower oil supplement. However, the study was under-powered (Section 4.4.7, p 182), and the mean tissue weights of females in the canola group were higher, if not significantly, than those of controls in both absolute and relative terms for all tissues weighed at both 7 and 28 days of age. It is conceivable, therefore, that canola oil has a similar, if lesser, effect to that of sunflower oil. The apparent differential effects of sunflower and canola oil in females may be attributable to their respective mix of fatty acids. One important difference between the two, as noted in Section 4.1.4, p 147, is that canola oil contains around 10 % ALA, an ω-3 fatty acid, whereas sunflower oil contains virtually none. A considerable number of animal studies have shown that ω-3 fatty acids have anti-obesity effects, and there is evidence of more moderate fat-lowering effects in studies on small human cohorts [Belchior *et al*, 2015; Buckley & Howe, 2010; Flachs *et al*, 2009]. However, most trials have been carried out using EPA and DHA [Lorente-Cebrián *et al*, 2013], which have much stronger effects than ALA [Flachs *et al*, 2009]. Evidence for the beneficial effects of ALA is less convincing [Lorente-Cebrián *et al*, 2013], though a recent study has reported that serum ALA is inversely related to adiposity in school-age children, and may therefore be protective against weight gain [Perng *et al*, 2015]. Any difference between the effects of ALA and EPA/DHA could be important, because the synthesis of EPA and (particularly) DHA from ALA is relatively inefficient in humans [Barceló-Coblijn & Murphy, 2009; Nichols *et al*, 2010], though possibly more efficient in infants than adults [Brenna *et al*, 2009].

Another key difference between sunflower and canola oil is that the former contains a higher percentage of LA, an ω-6 fatty acid (Section 4.1.2.2, p 147). A number of animal and *in vitro* studies have shown that, in contrast to ω-3 fatty acids, LA and (particularly) ARA,[36] have an adipogenic effect, and increase the deposition of fat through both hyperplasia and

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[36] ARA is the principal adipogenic ingredient of fetal bovine serum, which is a component of the 'cocktail' used to initiate adipocyte differentiation *in vitro* [Ailhaud *et al*, 2006].

hypertrophy. Indeed, it has been proposed that the rise in obesity in the western world over the last few decades, which has coincided with a decline in the intake of saturated fat, may be associated with an increase in the *per capita* intake of ω-6 fatty acids. However, there has been a lack of studies to confirm the effects of ω-6 fatty acids in humans [Ailhaud *et al*, 2006; Muhlhausler & Ailhaud, 2013].

These two opposing effects potentially provide a plausible explanation for the different effects of sunflower and canola oils on the adipose tissue of females, with the higher ω-6 content of the former favouring fat deposition, and the ω-3 content of the latter inhibiting it. However, there is a problem with this hypothesis. Firstly, unsaturated fatty acids are subject to biohydrogenation in the (ewe's) rumen, though some do survive to be absorbed intact by the small intestine [Alvarenga *et al*, 2015]. Secondly, even if a proportion of the supplemented LA and ALA is transferred intact from mothers to their offspring, they may not be sufficiently adipogenic or inhibitive respectively *per se* to account for the observed effects, and the efficiency of their conversion to the more complex, and probably more effective, fatty acids ARA, EPA and DHA is unknown. Considerably more work would be required to support this hypothesis, not least analysis of lipids in the mothers' milk.

None of the above explains why a sunflower oil supplement increases fat deposition in females, but not in males, though in general human females have more adipose tissue than males throughout life [Blaak, 2001; White & Tchoukalova, 2014], and a recent study reported that 11 week-old male lambs had a lower percentage carcass fat than females [Wallace *et al*, 2014]. This suggests that human and ovine females have a greater capacity to deposit fat than males, and indeed, numerous studies have reported sex-specific differences in various aspects of adipose tissue biology, including adipogenesis, metabolism and handling of fatty acids [White & Tchoukalova, 2014]. One potential mechanism for reduced adipogenesis in males could be a less efficient conversion of the ω-6 fatty acid LA to the more adipogenic ARA. While specific evidence for this could not be found, it has been reported that the ω-3 fatty acid ALA is more efficiently converted to EPA in human females than in males [Childs *et al*, 2014], and given that ω-3 and ω-6 fatty acids use the same desaturase and elongase enzymes [Barceló-Coblijn & Murphy, 2009; Lorente-Cebrián *et al*, 2013], it does not seem unreasonable to postulate a similar sex difference for conversion of LA. Furthermore, more general evidence for sexual dimorphism in ARA levels is reported in a recent systematic review, in which it was found that human males have lower levels of ARA than females in plasma lipids, though higher levels in adipose tissue [Lohner *et al*, 2013]. In any event, further work must be carried out to confirm the sex-specific effect on adipose tissue mass of maternal sunflower oil supplementation, and to ascertain the underlying mechanism.

4.5.2 Rectal temperature

4.5.2.1 The low rectal temperature at 7 days of age of male offspring of ewes fed a sunflower oil supplement may be attributable to reduced thermogenic activity

The ontogeny study in Chapter 3 established that expression of ATF2 and CIDEA, which were classified as thermogenic, did not decline with age in sternal adipose tissue (Section 3.4.2, p 134). These genes are therefore probably not primarily thermogenic in that tissue. Mean expression of the other four thermogenic genes, DIO2, PRLR, PGC1α and UCP1, which are more definitive markers of BAT, was lower in 7 day-old males in the sunflower group than in controls in all cases, although the result was only significant for DIO2. Mean UCP1 protein expression was also lower in 7 day-old males in the sunflower group, but again the difference was not significant. It is therefore possible that the lower rectal temperature of males in that group is a function of reduced thermogenic activity, but more work would be required to definitively establish such a link. However, all the included lambs had normal temperatures for their age, so the difference is unlikely to be biologically significant.

4.5.3 Plasma metabolites

4.5.3.1 A maternal sunflower or canola oil supplement during lactation has no effect on the plasma concentration of IGF1 in suckling lambs

Maternal supplementation of sunflower or canola oil had no effect on the offspring plasma concentration of IGF1 in either males or females at 7 or 28 days of age. The value measured was total IGF1 (as against free IGF1), which includes IGF1 bound to IGF binding proteins. Previous (human) studies that have examined the relationship between plasma IGF1 levels and BMI or fat mass have been inconsistent. While most report that circulating IGF1 levels are inversely correlated with BMI and/or fat mass [Galli *et al*, 2012; Pardina *et al*, 2010; Parekh *et al*, 2010], one large study using computed tomography (CT) scanning to measure adipose tissue found no relationship between IGF1 levels and BMI or total, abdominal or subcutaneous fat [Schoen *et al*, 2002]. Furthermore, one study (in men only) reports no difference in total IGF1 levels between obese and control groups, but found that free IGF1 is higher in the obese group [Nam *et al*, 1997], while another found that in females, but not in males, IGF1 levels were inversely associated with abdominal fat, but were not associated with BMI or any other measure of obesity in either sex [Rasmussen *et al*, 1994]. On the balance of evidence, therefore, one might have expected a reduction of circulating IGF in the female offspring of mothers fed the sunflower oil supplement to correspond with the increased fat mass, but any such difference was probably too small to be detected.

4.5.3.2 A maternal sunflower or canola oil supplement during lactation increases the levels of circulating leptin in female suckling lambs

Both sunflower and canola oil supplements given to lactating ewes increased the plasma leptin concentration of their female, but not male, suckling lambs. This apparent sexual dimorphism is relatively, though not entirely, consistent with the effects of maternal fat supplementation on offspring adipose tissue weight and gene expression at 28 (but not 7) days of age. Neither supplement had any effect on leptin mRNA expression in 28 day-old male lambs, but both supplements increased leptin mRNA expression in female lambs, though there was only weak evidence for the effect of sunflower oil. The increases in leptin plasma concentration and mRNA expression at 28 days are matched by a similar effect on fat mass for sunflower, though not canola, oil. However, there was a numerical increase in the mean fat mass of all depots tested for females in the canola group, and the study was considerably underpowered (Section 4.4.7, p 182). As suggested in Section 4.5.1.2, p 185, therefore, it is possible that canola oil has a similar, if less potent, effect to sunflower oil, on fat mass. In general, therefore, the sexual dimorphism observed in fat mass, leptin plasma concentration and leptin mRNA expression are similar, and this is not unexpected given that plasma levels of leptin and leptin gene expression generally correlate to WAT mass (Section 1.8.3.5, p 58) [Ahima & Flier, 2000; Friedman & Halaas, 1998].

4.5.4 Gene expression

4.5.4.1 Maternal fat supplementation may boost adipogenic and metabolic gene expression in the adipose tissue of female lambs, but the results are inconclusive

The hypothesis that fat supplements given to lactating ewes would increase the expression of adipogenic and metabolic genes in the adipose tissue of their suckling lambs was weakly supported, but only in female lambs. As shown in Figure 4.9, p 165, only FABP4 in the sunflower (S) group and leptin (LEP) in the canola (C) group were significantly upregulated at 28 days of age, with weak evidence of an effect for FABP4, PPARγ and INSR in the canola group and leptin in the sunflower group. However, numerically, the mean expression in females of all adipogenic and metabolic genes except NR3C1 (glucocorticoid receptor) and RIP140 was higher in both the sunflower and canola groups than in controls (N; Table 4.9, p 166), often substantially. Furthermore, the lipid droplet protein-encoding gene CIDEA was significantly upregulated in the canola group, with weak evidence of the effect in the sunflower group. Although this gene is classed as thermogenic, results from the ontogeny study (Section 3.4.2, p 134) suggest that it is not thermogenic in the sternal adipose tissue of sheep, and is therefore likely to assume a more adipogenic or metabolic role.

Although the evidence is weak, the upregulation of adipogenic and metabolic genes in females is consistent with the increase in relative adipose tissue mass observed in female offspring of ewes fed a sunflower oil supplement. If the increase in fat mass is attributable to both hyperplasia and hypertrophy, expression of genes involved in adipogenesis, fatty acid transport and lipid droplet formation would be expected to increase. There was no significant increase in relative adipose tissue mass in female offspring of ewes fed a canola oil supplement, so *prima facie* the upregulation of adipogenic and metabolic genes in the canola group is inconsistent with the tissue weight results. However, mean tissue weights for females were numerically higher than those of controls in all four depots tested, and the study was underpowered (Section 4.4.7, p 182). As suggested in Section 4.5.1.2, p 185, therefore, it is possible that canola oil has a similar, if less potent, effect to sunflower oil, but that the sample size was too small, or the timescale too short, to detect an increase in fat mass.

The possible effects of sunflower and canola oil on adipose tissue mass vis-à-vis their respective ω-6 and ω-3 fatty acid components were discussed in Section 4.5.1.2, p 185. It was suggested there that the apparent differential effects of maternal sunflower oil and canola oil supplementation on fat mass in female lambs could be attributed to the higher content of pro-adipogenic ω-6 fatty acids in the former and the lower content of anti-obesogenic ω-3 fatty acids in the latter. If that were the case, it might be expected that the expression of adipogenic and metabolic genes would be higher in female offspring of ewes fed a sunflower oil supplement than those of their canola oil-fed counterparts, which does not appear to be the case. This therefore appears to contradict the ω-6/ω-3 argument, but the mechanisms by which these fatty acids mediate their respective pro-adipogenic and anti-obesogenic effects are unknown. It is conceivable, for example, that ω-3 fatty acids act downstream of adipogenic and metabolic genes, and thereby suppress the increase in fat mass without affecting expression of those genes. In any event, the evidence is too weak to be definitive, and further work needs to be carried out to confirm the tentative findings before a potential mechanism can be determined.

4.5.4.2 A maternal sunflower or canola oil supplement during lactation has no effect on thermogenic gene expression in suckling lambs

The hypothesis that fat supplements given to lactating ewes would increase the expression of thermogenic genes in the adipose tissue of their suckling lambs was not supported. As suggested in Section 4.2, p 148, it was felt that there might be a compensatory mechanism whereby increases in fat mass in young lambs as a result of maternal fat supplementation would be partially offset by an increase in thermogenic activity. Although this idea might

seem to conflict with reports that BMI and obesity are inversely correlated with BAT in adult humans, at least in older individuals [Cypess *et al*, 2009; Saito *et al*, 2009; van Marken Lichtenbelt *et al*, 2009], the cause and effect nature of that relationship is unclear. Given that maternal fat supplementation only seems to increase fat mass and adipogenic and metabolic gene expression in female lambs, the original hypothesis would predict a similar sexual dimorphism in the expression of thermogenic genes.

Indeed, in males there was no significant difference in expression of thermogenic genes between supplemented groups and controls (N) at 28 days of age, and although there was weak evidence of an increase in DIO2 expression in the canola (C) group, this disappeared when the outlier discussed in Section 4.4.4, p 161, was removed. The picture was less clear at 7 days of age, with a significant reduction in DIO2 expression in the sunflower (S) group, and weak evidence of increased ATF2, CIDEA and UCP1 expression in the canola group. The ontogeny study suggests that ATF2 and CIDEA are probably not primarily thermogenic in the sternal adipose tissue of sheep (Section 3.5.2.2, p 142), and expression of the more definitive thermogenic genes was too varied to draw any firm conclusions.

With the exception of CIDEA, which is considered in Section 4.5.4.1, p 188, there were no significant differences in expression of thermogenic genes in females at 28 days of age, where the hypothesis would have predicted an increase (at least in the sunflower group). At 7 days of age, expression of DIO2, PGC1α, PRLR and UCP1 in the canola (C) group was lower numerically than that of controls in all cases, but the difference was only significant in UCP1, although there was weak evidence for the effect with PGC1α. There is therefore a faint suggestion that maternal canola oil supplementation might reduce thermogenic gene expression in the sternal adipose tissue of 7 day-old female lambs, which is in the opposite direction to the original hypothesis. Further work would be required to ascertain whether the effect is real or simply an anomaly, but as it seems to disappear by 28 days of age, it is probably not of great biological significance.

In summary, therefore, fat supplements given to lactating ewes do not seem to affect the expression of thermogenic genes in the sternal adipose tissue of their suckling lambs. However, it is worthy of note that a colleague, Miss Rachel Woods, has found that there is around double the amount of UCP1 protein in the perirenal adipose tissue of the 28 day-old lambs in the canola group than in that of controls (with no apparent sexual dimorphism) [Woods *et al*, 2015]. There was insufficient time to carry out protein analysis work on the 28 day-old sternal adipose tissue samples, but it would be interesting to undertake such work to ascertain whether the gene expression results are matched by protein expression, and whether the effects of maternal fat supplementation are tissue-specific.

4.5.4.3 A 28 day-old male lamb that was a very high outlier for UCP1 expression had low body weight and adipose tissue mass

As reported in Section 4.4.4, p 161, UCP1 expression for one 28 day-old male lamb in the canola (C) group was extremely high, and of the order that would be expected for a 7 day-old animal. Further investigation indicated that this animal was almost certainly a genuine biological outlier. It is therefore of particular interest, because a mechanism that delays the normal decline in UCP1 expression with age could ultimately have clinical implications in combating obesity. It was one of an MF twin pair, and the first point of note is that its female sibling was excluded from further analysis because it was growth restricted at birth (Section 4.3.1.9, p 153). The birth weight of the outlier itself was the second lowest of lambs sampled at 28 days of age, and the third lowest of all twin lambs (ignoring its excluded sibling). At 7 days of age it had the fifth lowest body weight of animals sampled at 28 days of age, suggesting some catch-up growth, but by 28 days of age it had the lowest body weight of all animals sampled at that time point. Furthermore, it had the second lowest perirenal and sternal adipose tissue weights, and the lowest omental, pericardial and combined adipose tissue weights, of the 27 animals sampled at 28 days of age. Finally, its rectal temperature at sampling was at the higher end of the range.

It is not possible, of course, to conclude that the low body weight and adipose tissue mass at sampling are the result of high UCP1 expression, particularly as the animal had a relatively low birth weight. All that can be said is that they are not inconsistent with the concept of greater energy expenditure as a result of increased thermogenic activity. The reason for the high UCP1 expression remains unknown, but the environmental influences during gestation that caused growth restriction in its sibling may have triggered some adaptation in the outlier.

4.5.4.4 Expression of adipogenic, metabolic and thermogenic genes is higher in the 7 day-old male offspring of canola oil-supplemented ewes than in the females

The common patterns of expression of most adipogenic, metabolic and thermogenic genes across the nutritional groups and sexes at 7 days of age are discussed in Section 4.4.4, p 161, and led to a determined effort to optimise normalisation. It seemed strange at the time that different categories of genes should behave in the same way, although in retrospect, if an increase in thermogenic activity were to partially compensate for an increase in fat mass, as hypothesised in Section 4.2, p 148, then the effect would be less surprising. However, that hypothesis was not supported at 28 days of age, by which time in any event the common patterns of expression had disappeared.

A particular feature of these common patterns of expression was that 10 of the 20 genes tested were more highly expressed in 7 day-old males than females in the canola (C) group, to the extent that there were two discrete sets of values with no overlap. As the separation between male and female data points was particularly wide with UCP1, and as our research group had a tried and tested antibody for its protein, it was decided to test UCP1 protein with a view to determining whether the sexual dimorphism in UCP1 mRNA expression was mirrored in its protein expression. In the event, the discrete separation of male and female data points in the canola group was only replicated in the protein if an outlier on the mRNA/protein correlation plot was removed, and even then, separation between male and female data points was much reduced. There is therefore insufficient evidence to conclude that the apparent sexual dimorphism in UCP1 mRNA expression found in the canola group is replicated in its protein expression. Furthermore, only one of the histology slides for males in the canola group was successful, so the results of UCP1 mRNA and/or protein expression are neither supported nor contradicted by the tissue morphology.

In summary, therefore, the disparity between males and females in the canola group in expression of various adipogenic, metabolic and thermogenic genes is not well supported by the limited work on protein expression and is hard to explain, particularly as there is no such disparity in fat mass at 7 days of age. Without further work, therefore, it is difficult to conclude that the effect is other than just a curious anomaly.

4.5.5 Protein expression and histology

4.5.5.1 UCP1 protein expression and sternal adipose tissue morphology is not well correlated in 7 day-old lambs

Overall there was a correlation between UCP1 mRNA and protein expression, albeit not particularly high, and the expression pattern across the feeding groups and sexes was not dissimilar to that of UCP1 mRNA, though the peaks and troughs were 'dampened' such that differences in mean expression between feeding groups were generally less pronounced. However, although the comparison was qualitative rather than quantitative, there seemed to be limited correlation between UCP1 protein expression and adipose tissue morphology. This is surprising, and may be a function of the heterogeneous nature of adipose tissue. In cutting tissue for qPCR and western blotting, efforts were made to ensure that slices were cut from adjacent parts of the original frozen sample. However, separate samples, fixed in formalin, were used for histology, and these may have been taken from a slightly different part of the excised adipose depot. In particular, the sternal adipose tissue depots (one on each side of the neck) contain large lymph nodes, which were removed after excision.

There is some interaction between adipose tissue and lymph nodes [Pond & Mattacks, 1995], and it is possible that the distance of a section of adipose tissue from the local lymph node is a factor in its gene expression and/or tissue morphology.

4.6 Summary and conclusions

A nutritional study was carried out on sheep in which lactating ewes were allocated to one of three feeding groups immediately after parturition: a control group, which was fed a standard diet; or one of two experimental groups, which were fed a standard diet with a small supplement of either sunflower oil or canola oil. Their (mainly twin) lambs were mother-fed for the duration of the study, and were sampled at either 7 or 28 days of age. The body and tissue weights of the lambs were measured, their rectal temperatures were taken, their plasma concentrations of IGF1 and leptin were measured, and expression of various adipogenic, metabolic, thermogenic and developmental genes was tested in their sternal adipose tissue. The expression of UCP1 protein in their sternal adipose tissue was also examined, in 7 day-old lambs only. It was hypothesised that sunflower and canola oil supplements fed to lactating ewes would increase the fat mass of their suckling lambs, and that this would be reflected in an increase in the expression of adipogenic, metabolic and thermogenic genes in their sternal adipose tissue.

Although the evidence was not always strong, it was generally found that the response in suckling lambs to maternal fat supplements was sex-specific, with most effects being seen in females, but not in males. The exception was rectal temperature, which was about 0.5 °C lower in 7 day-old males in the sunflower group than in controls. The hypothesis that maternal fat supplements would increase fat mass in suckling lambs was supported in the female offspring of ewes fed a sunflower oil supplement, but not of those fed a canola oil supplement. The apparent differential effects of the two supplements may be a function of their respective fatty acid constituents, with sunflower oil containing more ω-6 fatty acids, which are pro-adipogenic, and canola oil containing more ω-3 fatty acids, which are anti-obesogenic. There was some support in female lambs for the hypothesis that maternal fat supplements would increase expression of adipogenic and metabolic genes in adipose tissue, but the evidence was relatively weak and inconclusive. There was much stronger evidence that plasma leptin concentrations in females were higher in both supplemented groups than in controls. The hypothesis that maternal fat supplements would increase expression of thermogenic genes in adipose tissue was not supported in either males or females. At 28 days of age there was no difference in the expression of thermogenic genes between supplemented groups and controls, and at 7 days of age there was a suggestion

that expression in the sunflower group for males and canola group for females might be lower than that of controls, with the former correlating with the lower rectal temperature. The study was considerably underpowered, which may well be a factor in the poor quality of evidence, and further work would be required to confirm the sex-specific effects of fat supplementation.

It was also found that various adipogenic, metabolic and thermogenic genes, including UCP1, were more highly expressed in 7 day-old males in the canola group than in females, often substantially, with a discrete separation of data points between the sexes. However, this apparent dimorphism was not well supported by UCP1 protein analysis, the effect had disappeared by 28 days of age, and the reasons for it remain unclear. Finally, while there was a reasonable correlation between UCP1 mRNA and protein expression, a qualitative assessment indicated that UCP1 protein expression and tissue morphology was not that well correlated. This may be a function of the heterogeneous nature of adipose tissue, with samples for western blotting and histology possibly being taken from different parts of the excised adipose depot.

5 The effect of late gestational and postnatal diet on adipose tissue in 6 month-old lambs

5.1 Introduction

This study (Study B) forms part of a wider study on the effects of late gestational and early postnatal nutrition in sheep which is being conducted by the Department of Veterinary, Clinical and Animal Sciences at the University of Copenhagen [Khanal *et al*, 2015; Khanal *et al*, 2014]. The overall study looks at whether the effect of late gestational over- and under-nutrition on adiposity and metabolic and endocrine function is similar, and if subsequent exposure to a high-fat diet enhances any prenatal programming effects. This study looks specifically at the effect of modifications in late gestational and postnatal nutrition on the expression of adipogenic, metabolic, thermogenic and developmental genes in the sternal and subcutaneous adipose tissue of 6 month-old lambs. Sternal adipose tissue was selected for the reasons discussed in Section 1.6, p 51. It was also decided to examine subcutaneous adipose tissue following a recent bovine study which found that UCP1 gene expression was significantly higher in the subcutaneous depot of 30 month-old steers fed a high-energy, high-protein diet for 20 months than in that of roughage-fed controls [Asano *et al*, 2013].

5.2 Hypotheses

As explained in Section 1.4, p 38, fetal over- and undernutrition can both programme for the risk of adult obesity. Indeed, it has been proposed that the relationship between birth weight (often used as a proxy for the intrauterine nutritional environment) and adult fat mass is U- or J-shaped [McMillen & Robinson, 2005], and it has been suggested that there may be an optimal birth weight, dependent on the mother, at which the programming of obesity risk is minimised [Desai & Ross, 2011]. Furthermore, studies in sheep indicate that maternal over- and undernutrition lead to the same organ and tissue abnormalities in the fetus, and the same postnatal metabolic changes in offspring, possibly through epigenetic

changes to gene expression [Ford & Long, 2012]. It was therefore hypothesised that both late gestational over- and undernutrition would alter gene expression in adipose tissue.

It was expected that lambs on a high-fat postnatal diet would increase their fat mass relative to that of controls, and it was hypothesised that this would be by a combination of both hypertrophy and hyperplasia. It was also hypothesised, therefore, that the expression of adipogenic and metabolic genes in their adipose tissue would increase relative to that of controls. In the light of the findings by Asano *et al* [2013], it was further hypothesised that a high-fat diet would also increase the expression of thermogenic genes relative to that of controls, possibly in partial compensation for the predicted increase in fat mass.

The hypotheses for this chapter, therefore, were that:

- maternal over- and undernutrition in late gestation would change the expression of adipogenic, metabolic and thermogenic genes in the sternal and subcutaneous adipose tissue of 6 month-old lambs;

- a high-carbohydrate, high-fat (HCHF) postnatal diet would increase expression of adipogenic, metabolic and thermogenic genes in the sternal and subcutaneous adipose tissue of 6 month-old lambs.

5.3 Materials and methods

5.3.1 Animal husbandry and interventions

5.3.1.1 Overview

The experimental protocol has been published [Khanal *et al*, 2014], but is reproduced in detail below. Essentially it is a 3 × 2 factorial model, with pregnant ewes being divided into three feeding groups in late gestation (overnourished, undernourished and control), and their twin offspring each being allocated to one of two feeding groups postnatally (HCHF and control). Work was carried out under the auspices of the Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark. All experimental procedures were approved by the Danish National Committee on Animal Experimentation.

5.3.1.2 Selection, location and housing

A number of three year-old Texel ewes from a private herd at a commercial farm on the island of Lolland, Denmark, were mated in the same week in November 2010 with eight Texel rams of the same breed and with similar inherited characteristics.[37] Pregnant ewes,

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[37] No information is available on the estimated breeding values (EBVs) of the rams.

in their second parity, were subsequently scanned on 10 February 2011 to ascertain fetal age and number. Following scanning, 36 twin-pregnant ewes were transferred to another commercial farm, Rosenlund Farm, Lynge, near Copenhagen, Denmark, for the duration of the study. They were moved to the latter farm one week before the start of the experiment so that they could adapt to indoor environmental conditions. They were housed in a barn in individual pens of approximately 1.5 m × 1.5 m, separated with rails and bedded with sawdust. The lambs were housed in the same barn as the ewes. They were separated from their mothers and siblings after 3 days and assigned to individual pens of 1.5 m × 0.75 m. At two months of age they were moved to larger pens of 1.5 m × 1.5 m.

5.3.1.3 Allocation of ewes and lambs to feeding groups

On arrival at Rosenlund Farm, ewes were given an immediate 20 ml subcutaneous injection of vitamins, being 10 ml Adesan and 10 ml Becoplex (Boehringer Ingelheim, Copenhagen, Denmark). They were fed a normal diet for one week and were then allocated to one of three feeding groups (Figure 5.1, p 198) for the last 6 weeks of gestation (term = 21 weeks): U (undernutrition; n = 14); N (control or 'normal' nutrition; n = 9); or O (overnutrition; n = 13). Allocations were made so as to ensure, as far as possible, an even distribution of body weight and body condition score (BCS) over the three groups.

The 72 offspring (36 male, 36 female) all received colostrum within 3 hours of birth and continued to feed from their mothers for the first three days postpartum (days 0–2). Male lambs were not castrated. From day 3 until 6 months of age (post puberty), the two lambs from each twin pair were fed differently, each being allocated to one of two postnatal feeding groups (Figure 5.1, p 198): C (control or 'conventional'; n = 36: 17 male, 19 female); or H (HCHF; n = 36: 19 male, 17 female). Allocations were made immediately after birth with a view to ensuring as uniform a distribution as possible by sex and body weight in the two postnatal groups derived from each late gestational group. The average birth weight by postnatal feeding group is shown in Figure 5.5, p 205.

The combination of three late gestational and two early postnatal feeding groups gives rise to six combined prenatal/postnatal treatment groups. These are identified by a two-letter code, the first letter representing the late gestational group and the second letter representing the postnatal group (Figure 5.1, p 198): UC, UH, NC, NH, OC and OH. These codes are used where applicable in subsequent figures and tables. Two male lambs died from natural causes within two weeks of birth, one from the UC group and the other from the UH group. The two lambs were not siblings, so the U late gestational group contains an 'unmatched' pair of singleton lambs. These deaths reduced the total number of lambs to 70 (34 males, 36 females). The distribution of the 70 lambs between treatment groups (where
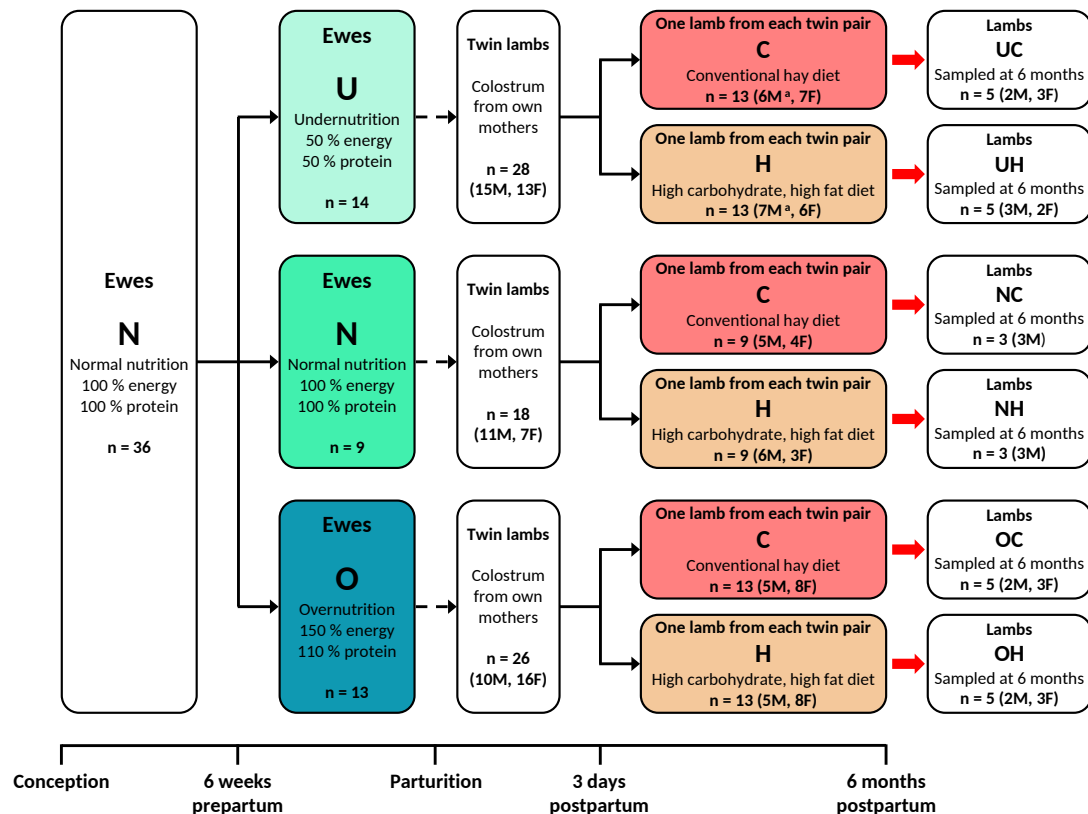
Figure 5.1 Experimental protocol. The experiment is a 3 × 2 factorial design with 3 late gestational nutrition groups and 2 early postnatal nutrition groups. For the first 15 weeks of gestation (term = 21 weeks), all 36 twin-pregnant ewes were fed a 'normal' diet. For the last 6 weeks of gestation, the ewes were divided into 3 nutrition groups: O (overnutrition), 150 % and 110 % of normal requirements for energy and protein respectively; N ('normal' nutrition), normal requirements for energy and protein as determined by the US National Research Council [NRC, 2007]; U (undernutrition), 50 % of normal requirements for energy and protein. For the first 3 days after birth, all lambs were fed on their own mother's colostrum. After day 3, the lambs were divided into 2 nutrition groups: C (conventional), hay and milk replacer until 8 weeks of age, and hay only thereafter; H (high-carbohydrate, high-fat), rolled maize and a 1:1 mix of dairy cream and milk replacer. The twin lambs were allocated to their nutrition groups immediately after birth with a view to ensuring as uniform a distribution as possible by sex and birth weight in the two postnatal nutrition groups derived from each late gestational group. A subsection of the lambs was humanely euthanased and sampled at 6 months of age, with the others continuing on a new feeding programme with a view to further study at 2 years of age. M is male and F is female. The colour coding is intended to facilitate interpretation of the results figures.

^a Two non-sibling male lambs from the same late gestational nutrition group (U) but different postnatal groups died of natural causes within a few weeks of birth.

M is male and F is female) is: UC, n = 13 (6M, 7F); UH, n = 13 (7M, 6F); NC, n = 9 (5M, 4F); NH, n = 9 (6M, 3F); OC, n = 13 (5M, 8F); and OH, n = 13 (5M, 8F).

5.3.1.4 Feeding

The diet for the N group was designed to fulfil 100 % of the daily requirements for metabolisable energy (ME) and crude protein in sheep as specified by the (US) National Research Council [NRC, 2007], and contained artificially dried green hay (Dangrønt, Ølgod, Denmark)

supplemented by a commercial concentrate (Fårefoder F; NAG, Helsinge, Denmark). The diet for the U group was designed to provide 50 % of daily ME and protein requirements and comprised 50 % of the hay and concentrate given to the N group. Small amounts of barley straw, which provides a minimal contribution to energy and protein intake, were also provided to ewes in the U group, to prevent discomfort from hunger. The diet for the O group was designed to provide 150 % and 110 % respectively of the daily requirements for ME and crude protein, and consisted of the same amount of hay and concentrate fed to ewes in the U group, supplemented by whole barley (Rosenlund Farm, Lynge, Denmark) to bring it up to the required energy and protein level. The ewes were fed in two meals, with half the daily ration being provided at each meal. Water was made available *ad libitum*, and 10 g of a mineral mix (Får Min; Dansk Vilomix, Mørke, Denmark) was given to all ewes with each meal. After parturition, ewes were provided with green hay *ad libitum* plus a daily supplement of 300 g concentrate and 300 g barley in two meals, to ensure sufficient production of colostrum. The bodyweight, BCS and energy and protein intake of the ewes in each group for the last six weeks of gestation are shown in Table 5.1, p 200. The chemical composition and energy content of the feeds are shown in Table 5.2, p 200.

Lambs in the C group were fed milk replacer (180 g milk powder per litre; Elitemilk Lamb; DLA Group, Galten, Denmark) until eight weeks of age, four times a day from a bottle on days 3–7, then twice daily from a suckling bucket thereafter. From day 14 they were also fed good quality hay (Dangrønt) in two daily meals. Daily milk and hay allowances were adjusted on a weekly basis to ensure that lambs gained live weight at a rate of about 225 g per day. Lambs in the H group were fed a 1:1 mixture of milk replacer and dairy cream (Osted Ost og Mejeri, Lejre, Denmark) *ad libitum* up to a maximum of 2.5 l per day, four times a day from a bottle on days 3–7, then twice daily thereafter from a suckling bucket. In addition, they were fed rolled maize (Majs R2 Flakes; R2 Agro A/S, Hedensted, Denmark) *ad libitum* up to a maximum of 1 kg per day in two meals. All lambs also received a small amount of barley straw each day, water *ad libitum*, and the recommended amount of vitamin-mineral mix [NRC, 2007]. The chemical composition and energy content of the feeds are shown in Table 5.2, p 200. The energy and protein intake of the lambs for each of the six prenatal/postnatal treatment groups are shown in Table 5.3, p 201. The HCHF diet was designed so that as much ingested food as possible would avoid fermentation in the rumen and be digested in the stomach or intestine, as with monogastrics. Delivery of the milk-cream mixture via a suckling bucket fitted with a rubber teat elicits the oesophageal groove reflex, which allows the liquid to flow directly to the stomach via the oesophageal groove and omasal canal, bypassing the rumen and reticulum [Comline & Titchen, 1951; Ørskov & Benzie, 1969] (Figure 5.2, p 201). Furthermore, degradation of maize starch in the

Table 5.1 Body weight, body condition score (BCS) and daily energy and protein intake of twin-pregnant sheep during the last 6 weeks of gestation. Table adapted from Khanal *et al* [2014]. Term = 147 days. Late gestational feeding groups are: overnutrition (O); 'normal' nutrition (N) and undernutrition (U). BCS is in arbitrary units (AU) and on a scale of 1 (very lean) to 5 (very fat). Data shown for body weight, BCS, digestible energy (DE) and digestible crude protein (DCP) are least square means ± SEM. Data within rows with different superscript letters are significantly different (p < 0.05).

Item	Late gestational feeding groups		
	U	N	O
Number of sheep	14	9	13
Body weight 6 weeks prepartum (kg)	75.1 ± 1.5	77.6 ± 1.9	76.3 ± 1.5
Body weight just before parturition (kg)	79.4 ± 1.4 [a]	89.7 ± 3.2 [b]	92.5 ± 2.1 [b]
BCS 6 weeks prepartum (AU)	3.64 ± 0.10	3.69 ± 0.12	3.87 ± 0.10
BCS just before parturition (AU)	2.79 ± 0.12 [a]	3.75 ± 0.06 [b]	4.31 ± 0.06 [c]
DE 6 weeks prepartum (MJ d^{-1})	8.70	17.8	20.8
Average DE for last 6 weeks prepartum (MJ d^{-1})	11.0 ± 0.6 [a]	22.9 ± 0.8 [b]	34.8 ± 0.6 [c]
DE just before parturition (MD d^{-1})	11.7	24.3	39.4
DCP 6 weeks prepartum (g d^{-1})	99.7	104	199
Average DCP for last 6 weeks prepartum (g d^{-1})	125 ± 4.6 [a]	263 ± 5.7 [b]	284 ± 4.5 [c]
DCP just before parturition (g d^{-1})	134	278	311

Table 5.2 Chemical composition and energy content of feeds. Table adapted from Khanal *et al* [2014]. DM, dry matter; ANDF, amylase-treated neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; CF, crude fat; DE, digestible energy.

Feeds	DM %	Ash % of DM	ANDF % of DM	ADF % of DM	ADL % of DM	CP % of DM	CF % of DM	DE MJ (kg DM)$^{-1}$
Sheep diet								
Hay	91.4	5.6	47.7	27	3.1	20.8	4.8	13.7
Barley	89.0	2.3	14	6	1.1	12.5	3.1	17.1
Concentrate	87.7	7.7	25.8	18	2.8	15.3	3.8	12.8
Lamb diet								
Hay	93.1	6.8	50.4	32.3	3.5	19.1	3.7	13.5
Maize	89.5	0.6	4.1	< 5	0.9	8.5	1.9	16.3
Milk replacer	95.6	7.1				22.5	23.6	19.2
Cream	42.9	0.8				4.3	38.0	30.5

rumen is slower than that of other cereals, allowing a substantial part of it to be digested in the intestine and absorbed as glucose [Ørskov, 1986; Ørskov *et al*, 1969].

Table 5.3 Daily energy and protein intake of lambs from 3 days to 6 months of age. Table adapted from Khanal *et al* [2014]. Groups are denominated by a two letter code, the first letter representing the late gestational feeding group of the mothers (overnutrition (O); 'normal' nutrition (N); undernutrition (U)), and the second letter representing the postnatal feeding group of the lambs (control (C); high-carbohydrate, high-fat (HCHF, H)). The HCHF lambs had a significantly higher intake of energy and lower intake of protein until 8 weeks than those on a control diet ($p < 0.0001$). From 8 weeks to 6 months, lambs on a control diet had a significantly higher intake of protein than HCHF animals ($p < 0.0001$). M is male, F is female. Data shown for digestible energy (DE) and digestible crude protein (DCP) are least square means ± SEM. Data within rows with different superscript letters are significantly different ($p < 0.05$).

Item	Prenatal/postnatal feeding group					
	UC	UH	NC	NH	OC	OH
Total number of lambs (M/F)	6/7	7/6	5/4	6/3	5/8	5/8
Sampled at 6 months (M/F)	2/3	3/2	3/0	3/0	2/3	2/3
Average DE (MJ d^{-1})						
3 days to 8 weeks	4.3±0.4 [a]	7.7±0.4 [b]	4.5±0.5 [a]	8.4±0.5 [b]	4.3±0.4 [a]	8.0±0.4 [b]
8 weeks to 6 months	11.7±0.7	10.9±0.7	11.9±0.9	12.5±0.9	12.4±0.7	11.5±0.7
Average DCP (g d^{-1})						
3 days to 8 weeks	40.5±1.3 [a]	17.2±1.3 [b]	42.9±1.6 [a]	18.1±1.6 [b]	41.2±1.3 [a]	17.2±1.3 [b]
8 weeks to 6 months	120±7.1 [a]	26.7±7.1 [b]	122±8.6 [a]	29.8±8.6 [b]	129±7.1 [a]	27.2±7.1 [b]

Figure 5.2 Suckling bucket used to feed milk-cream to lambs. The delivery of milk-cream via a suckling bucket fitted with a rubber teat elicits the oesophageal groove reflex, which allows liquid to flow directly to the stomach via the oesophageal groove and omasal canal, bypassing the rumen and reticulum [Comline & Titchen, 1951; Ørskov & Benzie, 1969]. The other bucket contains rolled maize. Photograph taken by Dr Prabhat Khanal (University of Copenhagen).

5.3.1.5 Metabolic challenges

At the age of 6 months, the lambs were subjected to a number of metabolic challenges (glucose, insulin and propionate), after which their blood was sampled at timed intervals. The results of these interventions do not form part of this study, and have been reported elsewhere [Khanal *et al*, 2015].

5.3.1.6 Tissue sampling

Following a 6-day recovery period after the last of the metabolic challenges, 26 lambs (UC, n = 5 (2M, 3F); UH, n = 5 (3M, 2F); NC, n = 3 (3M, 0F); NH, n = 3 (3M, 0F); OC, n = 5 (2M, 3F); and OH, n = 5 (2M, 3F)) were transferred to appropriate facilities at the Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark, and humanely euthanased. The 44 surviving lambs continued in the programme under a new feeding protocol with a view to further experiments and tissue sampling as adults. These later experiments do not form part of this study. Selection of lambs for sampling from each treatment group was made so that there was as even a distribution of body weight and sex as possible between sampled animals and survivors in each group, with the additional proviso that only one lamb from any twin pair was sampled. The exception to this is in the NC and NH groups, where a deliberate decision was made to sample males only because of the small number of females in these groups. This will allow a balance of male and females in these groups for future studies, but results in a sex imbalance in lambs sampled at six months of age. The sampled lambs were weighed, anaesthetised with $0.5 \, \mathrm{ml \, kg^{-1}}$ body weight of $10 \, \mathrm{g \, ml^{-1}}$ propofol (B Braun, Melsungen, Germany) by intramuscular injection, then sacrificed by decapitation. The carcasses were immediately dissected by a team from the University of Copenhagen, and all major tissues and organs were collected, weighed and preserved, including sternal, epicardial, perirenal, mesenteric and subcutaneous (from above the *longissimus dorsi* muscle) adipose tissue. A portion of adipose tissue samples snap-frozen in liquid nitrogen was subsequently despatched in dry ice to the University of Nottingham for the purposes of this study.

5.3.2 Laboratory procedures

The procedures for determining gene expression are described in Section 2.4, p 70.

5.3.3 Statistics

The overall methodology for statistical analysis and presentation of data is described in Section 2.7, p 128. Results for body weight, tissue weights and fat deposition patterns have

been previously published [Khanal *et al*, 2014]. The authors of that paper used a general linear model and presented their results in terms of 'least square means'. Least square means take into account the effect of other variables in a model and arguably, therefore, provide a better estimate of the true population mean. In this thesis, however, a linear model has not been used, as the data did not appear to be normally distributed, and results are therefore presented as simple arithmetic means. In most cases the underlying findings are similar, and any material differences are noted below. All three prenatal groups were compared (by Kruskal-Wallis test), as all three pairwise comparisons (UN, UO and NO) were relevant. The direct UO comparison was of interest with a view to determining whether late gestational over- and undernutrition would have similar effects on gene expression.

Statistical power calculations were only performed for tests by postnatal feeding group. In determining effect sizes for power calculations on tests of tissue weights, the absolute percentage differences between the means of the HCHF group (H) and controls (C), and the coefficients of variation for each group, were averaged across all tissues. In determining effect sizes for gene expression, the absolute percentage differences between the means of the H and C groups, and the coefficients of variation for each group, were averaged across all genes and both tissues (sternal and subcutaneous).

5.4 Results

There is an issue in analysing the results by sex, in that no females from the prenatal control (N) group were sampled (Section 5.3.1.6, p 202). Furthermore, the results for male and female lambs in terms of both body and tissue weights and gene expression were very similar in most cases. Results are therefore presented mainly on a combined sex basis. Any significant variation between the two sexes is noted in the main text, and detailed results for the individual sexes have been included in Appendix D.

5.4.1 Body and tissue weights

5.4.1.1 Birth weight

For the combined sexes, the mean birth weight of the undernutrition (U) group (3.85 ± 0.16 kg; n = 26) was significantly lower by some 0.5 kg than those of the 'normal' (N) group (4.35 ± 0.12 kg; n = 18) and overnutrition (O) group (4.39 ± 0.14 kg; n = 26) (p = 0.0248 and 0.0201 respectively). This finding has previously been reported by Khanal *et al* [2014]. However, further analysis reveals that the difference is entirely attributable to male lambs (Figure 5.3, p 204). The mean birth weights of male lambs in the U group were 0.8–1.0 kg lower than those in the N and O groups (p = 0.0088 and 0.0080 respectively), whereas the
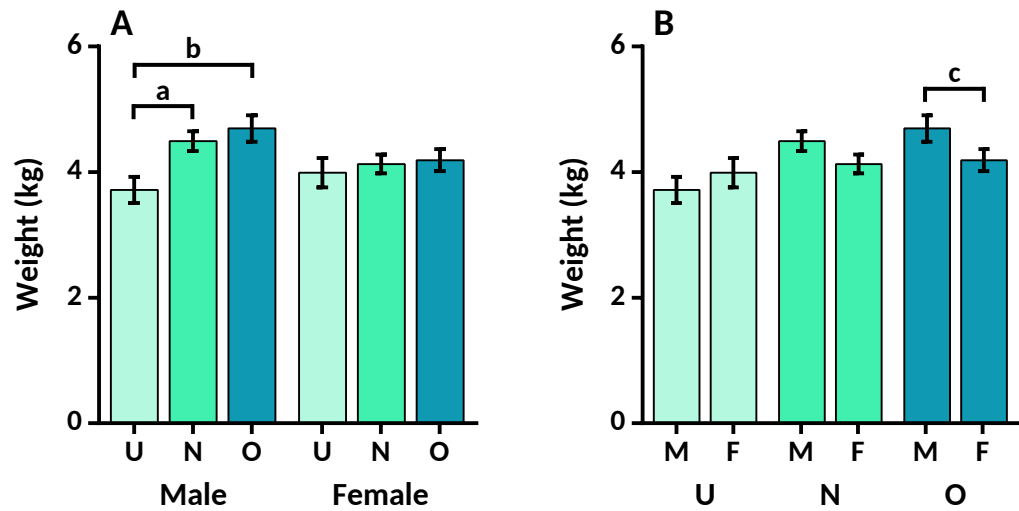
Figure 5.3 Birth weights by prenatal feeding group and sex. The same data are presented as (**A**) feeding group within sex and (**B**) sex within feeding group. Values shown are mean birth weight ± SEM. Feeding groups are: undernutrition (U: n = 26; 13M, 13F); 'normal' nutrition (N: n = 18; 11M, 7F); overnutrition (O: n = 26; 10M, 16F); M is male, F is female. The three prenatal feeding groups within each sex were compared by Kruskal-Wallis test (M: p = 0.0067; F: p > 0.1) followed by *post hoc* Dunn's tests where applicable (a: p = 0.0248; b: p = 0.0027). No adjustments have been made to p-values for multiple comparisons. The two sexes within each feeding group were compared by Mann-Whitney U-test (c: p = 0.0231).
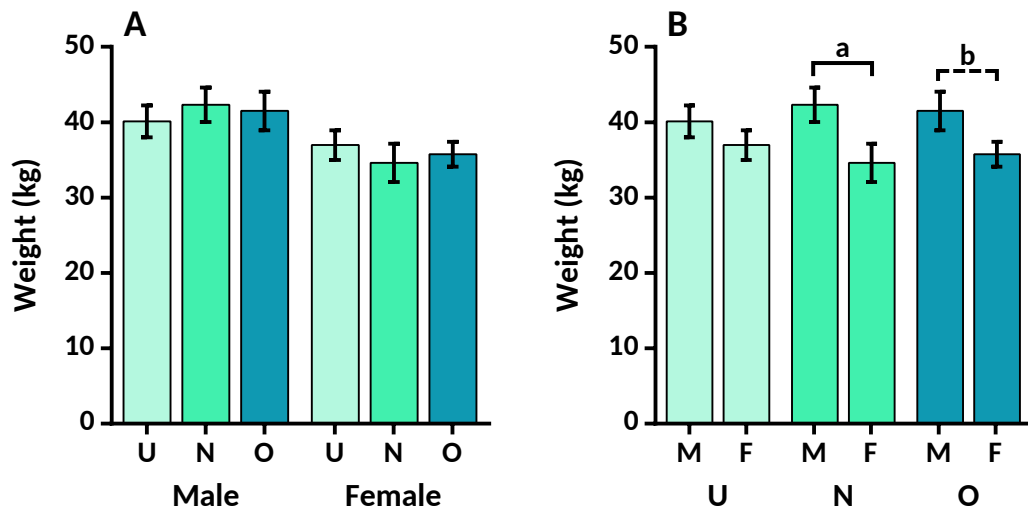
Figure 5.4 Body weights at six months by prenatal feeding group and sex. The same data are presented as (**A**) feeding group within sex and (**B**) sex within feeding group. Values shown are mean body weight ± SEM. Feeding groups are: undernutrition (U: n = 26; 13M, 13F); 'normal' nutrition (N: n = 18; 11M, 7F); overnutrition (O: n = 26; 10M, 16F); M is male, F is female. The three prenatal feeding groups within each sex were compared by Kruskal-Wallis test (p > 0.1). The two sexes within each feeding group were compared by Mann-Whitney U-test (a: p = 0.0415; b: p = 0.0577).

mean birth weights of female lambs varied by only 0.2 kg across the three groups, and the differences were not significant (p > 0.1). There was no significant difference (p > 0.1) between the mean birth weights of males (4.26 ± 0.13 kg; n = 34) and females (4.11 ± 0.12 kg; n = 36) overall, or in the U and N groups. However, males in the O group were significantly heavier than females by about 0.5 kg (p = 0.0231; Figure 5.3, p 204).

5.4.1.2 Body weight at six months of age

The pre-fast body weights of all lambs (n = 70) were recorded at six months of age, just before they were subjected to metabolic testing. For the two sexes combined, there was no significant difference (p > 0.1) between the mean body weights of the prenatal groups (U: 38.5 ±1.5 kg, n = 26; N: 39.3 ± 1.9 kg, n = 18; O: 38.0 ± 1.5 kg, n = 26). This conflicts with the results published by Khanal *et al* [2014], where lambs in the N group were significantly heavier than those in the O group, but the authors of that paper only analysed the subset of lambs that were sampled at 6 months (n = 26). Further analysis reveals that there is no significant difference (p > 0.1) between the mean body weights of the prenatal groups by sex (Figure 5.4, p 204). Overall, males (41.2 ± 1.3 kg, n = 34) were significantly heavier than females (36.0 ± 1.1 kg, n = 36) at six months of age (p = 0.0014). This was reflected in the N group (p = 0.0415) and there was weak evidence for the effect in the O group (p = 0.0577). However, there was no significant difference (p > 0.1) between the mean body weights of males and females in the U group at six months of age (Figure 5.4, p 204).

The mean birth weights and body weights at six months by postnatal feeding group are shown in Figure 5.5, below. The mean birth weights are a function of allocation rather than
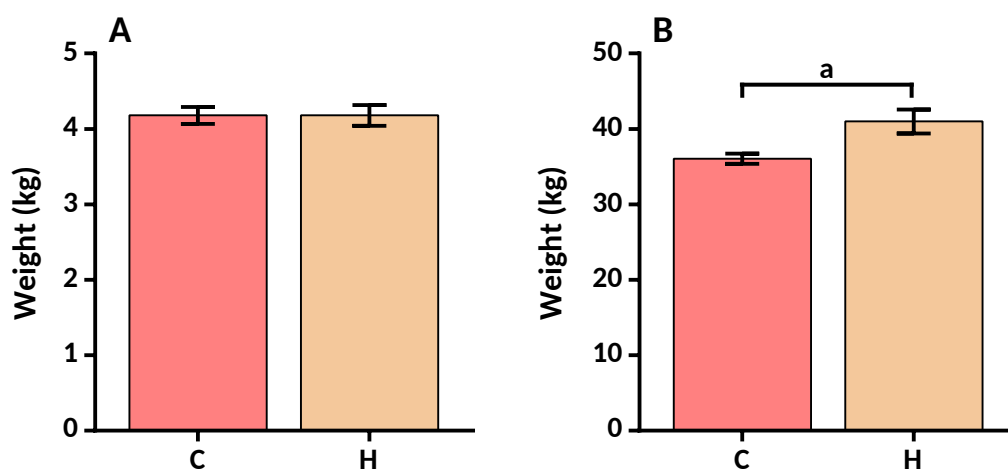
Figure 5.5 Birth weights and body weights at 6 months by postnatal feeding group. Values shown are (**A**) mean birth weights and (**B**) mean body weights at 6 months ± SEM of lambs by post-natal feeding group. Feeding groups are: control (C: n = 35; 16M, 19F); high-carbohydrate, high-fat (HCHF) (H: n = 35; 18M, 17F); M is male, F is female. The two groups were compared by Mann-Whitney U-test (a: p = 0.0176).

experimental outcome, and are identical. At six months of age, however, the body weight of the HCHF group was significantly higher (by some 5 kg) than that of the control group (p = 0.0176), which accords with the results reported by Khanal *et al* [2014]. A similar pattern was seen in both male and female lambs, but the differences were not significant for the individual sexes (Appendix D, Table D-1, p 289), although there was weak evidence for an effect in males (p = 0.0600).

5.4.1.3 Adipose tissue weights at six months of age

There were no significant differences in adipose tissue weights between prenatal feeding groups (p > 0.1), in either absolute terms (Table 5.4, below) or relative to body weight (Table 5.5, below). For three of the four adipose tissue depots measured, this is consistent with the findings of Khanal *et al* [2014]. However, the linear model approach used in that paper found that subcutaneous adipose tissue deposition was significantly higher in the 'normal' nutrition (N) group than in the overnutrition (O) group, both in absolute terms

Table 5.4 Absolute adipose tissue weights at 6 months by prenatal feeding group. Values shown are mean tissue weights ± SEM by depot and prenatal feeding group. Feeding groups are: undernutrition (U: n = 10; 5M, 5F); 'normal' nutrition (N: n = 6; 6M, 0F); overnutrition (O: n = 10; 4M, 6F); M is male, F is female. All groups were compared by Kruskal-Wallis test (p > 0.1 in all cases).

Tissue	Adipose tissue weight (g)		
	U	N	O
Mesenteric	749 ± 182	916 ± 406	698 ± 179
Perirenal	1 079 ± 327	975 ± 400	879 ± 244
Sternal	104 ± 25	116 ± 37	93 ± 23
Subcutaneous	187 ± 52	259 ± 91	133 ± 34
Combined	2 120 ± 572	2 265 ± 895	1 803 ± 471

Table 5.5 Relative adipose tissue weights at 6 months by prenatal feeding group. Values shown are mean tissue weights ± SEM relative to body weight (BW) by depot and prenatal feeding group. Feeding groups are: undernutrition (U: n = 10; 5M, 5F); 'normal' nutrition (N: n = 6; 6M, 0F); overnutrition (O: n = 10; 4M, 6F); M is male, F is female. All groups were compared by Kruskal-Wallis test (p > 0.1 in all cases).

Tissue	Adipose tissue weight (g per kg BW)		
	U	N	O
Mesenteric	18.3 ± 4.0	17.9 ± 6.8	18.7 ± 4.4
Perirenal	25.9 ± 7.4	19.4 ± 7.1	24.5 ± 6.9
Sternal	2.58 ± 0.53	2.40 ± 0.64	2.45 ± 0.54
Subcutaneous	4.51 ± 1.10	5.27 ± 1.59	3.57 ± 0.81
Combined	51.3 ± 12.7	45.0 ± 15.3	49.2 ± 12.3

and when expressed relative to body weight (p = 0.01 and p = 0.02 respectively). Results for the two individual sexes, which are shown in Appendix D, Table D-2, p 290 (absolute), and Table D-3, p 290 (relative), also revealed no significant differences (p > 0.1).

In regard to postnatal feeding groups, fat deposition was substantially and significantly higher (p < 0.0001) in the HCHF group (H) than in the control group (C) for all four adipose tissue depots sampled, both in absolute (Table 5.6, below) and relative (Figure 5.6, below) terms, which agrees with the findings of Khanal *et al* [2014]. Relative to body weight, the mean weight of mesenteric, perirenal, sternal and subcutaneous adipose tissue was 4½-, 9-, 4- and 5-fold higher respectively in the HCHF group than in controls. The combined mean relative weight of the four tissues was about 6-fold higher in the HCHF group (C: 13.8 ± 1.8; H: 84.3 ± 4.1 g per kg BW). The overall pattern was reflected in the results for the two sexes,

Table 5.6 Absolute adipose tissue weights at 6 months by postnatal feeding group. Values shown are mean tissue weights ± SEM. Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF; H: n = 13; 8M, 5F); M is male, F is female. The two groups within each tissue were compared by Mann-Whitney U-test (p < 0.0001 in all cases).

Tissue	Adipose tissue weight (g)	
	C	H
Mesenteric	238 ± 35	1 298 ± 150
Perirenal	169 ± 22	1 787 ± 136
Sternal	36.5 ± 2.5	168.3 ± 14.8
Subcutaneous	51.7 ± 5.4	314.4 ± 35.8
Combined	495 ± 61	3 567 ± 282
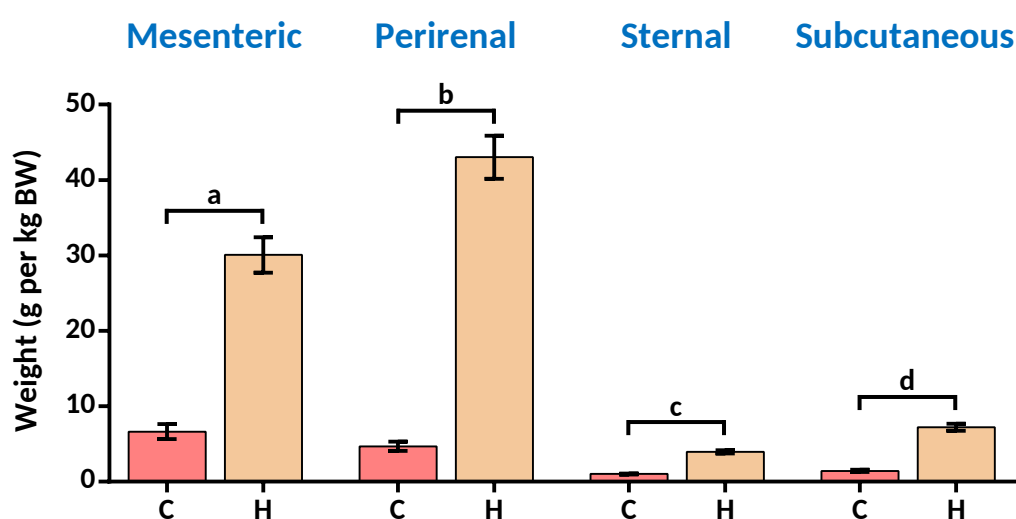
Figure 5.6 Relative adipose tissue weights at 6 months by postnatal feeding group. The values shown are mean tissue weights ± SEM relative to body weight (BW). Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF) (H: n = 13; 8M, 5F); M is male, F is female. The two groups within each tissue were compared by Mann-Whitney U-tests (a–d: p < 0.0001).

which are shown in Appendix D, Table D-4, p 291 (absolute) and Table D-5, p 291 (relative), with the differences remaining highly significant in every case (p = 0.0003 for males and p = 0.0043 for females in all tissues). Female lambs in the HCHF group had significantly more perirenal adipose tissue in relative terms than males (p = 0.0186), which is consistent with the findings of Khanal *et al* [2014]. There were no other significant sex effects on absolute or relative adipose tissue weight.

5.4.2 Gene expression

Gene expression was tested in two adipose tissues, sternal and subcutaneous, though the dataset was slightly reduced in the latter case. One sample of subcutaneous adipose tissue from a male lamb in the undernutrition (U) group yielded insufficient RNA for further analysis (n in the U group reduced from 10 to 9 overall, and from 5 to 4 for male lambs). Another subcutaneous sample from a female lamb in the overnutrition (O) group could not be tested with all genes as a result of accidental loss of cDNA (n in the O group reduced, in some cases, from 10 to 9 overall, and from 6 to 5 for female lambs).

The gene expression results are presented in terms of the relative expression of one feeding group to another in arbitrary units (AU), but in order to give an indication of how highly each gene is expressed in absolute terms, the mean C_T value and expression ranking of each gene in sternal and subcutaneous adipose tissue are shown in Table 5.7, p 209. The figures are not definitive, as the amplification efficiencies of primers vary, and there is a degree of subjectivity in selecting the quantification threshold. In general, the ranking of expression was very similar in both tissues. FABP4, adiponectin and PPARγ in that order were the most highly expressed of the genes tested, while thermogenic and developmental genes tended to be the least highly expressed. Indeed, the expression of some thermogenic and developmental genes was barely detectable and could not be reliably quantified, due to a combination of poor replicates (arising from sampling error at low expression levels) and background 'noise'.

5.4.2.1 Prenatal feeding groups

There were no significant differences between any of the three prenatal feeding groups in either sternal (Table 5.8, p 210) or subcutaneous (Table 5.9, p 211) adipose tissue (p > 0.1). Results by sex are shown in Appendix D, Table D-6, p 292 (sternal male), Table D-7, p 293 (sternal female), Table D-8, p 294 (subcutaneous male) and Table D-9, p 295 (subcutaneous female). There were no significant differences between groups, except in some cases for ADIPOQ and PGC1α in the sternal adipose tissue of males (Table D-6, p 292).

Table 5.7 Mean C_T values of genes of interest in sternal and subcutaneous adipose tissue. The mean C_T values from qPCR give an indication of the relative level of expression of the different genes, but are not definitive as there is a degree of subjectivity in selecting the quantification threshold. The values are logarithmic to the base 2, so a gene with a C_T value of 16 is approximately 2^{16} (ie 65 536) times more highly expressed than a gene with a C_T value of 32. The genes are ranked for each tissue, with the highest expressed being 1 and the lowest expressed being 20.

Category	Gene	Sternal		Subcutaneous	
		Mean C_T	Rank	Mean C_T	Rank
Adipogenesis	CEBPα	24.2	4	24.6	6
	NR3C1	24.8	5	23.7	5
	PPARγ	23.4	3	21.7	3
	SREBF1	27.4	11	26.6	10
Metabolism	ADIPOQ	18.6	2	17.9	2
	FABP4	15.3	1	15.8	1
	GPR120	29.0	12	28.7	12
	INSR	26.1	8	24.8	8
	LEP	24.9	6	25.1	9
	RIP140	27.1	10	24.8	7
Thermogenesis	ATF2	25.8	7	23.6	4
	CIDEA	30.1	13	31.0	15
	DIO2	34.0	18	33.7	18
	PGC1α	31.4	15	30.4	14
	PRLR	33.1	17	32.6	17
	UCP1	31.7	16	35.1	20
Development	HOXC9	26.8	9	26.9	11
	LHX8	37.3	20	31.2	16
	PRDM16	34.8	19	34.2	19
	SHOX2	31.0	14	29.7	13

5.4.2.2 Postnatal feeding groups

The results for sternal adipose tissue by postnatal feeding group are shown in Figure 5.7, p 212 (adipogenic genes), Figure 5.8, p 213 (metabolic genes) and Figure 5.9, p 214 (thermogenic and developmental genes). The results for subcutaneous adipose tissue are shown in Figure 5.10, p 215 (adipogenic genes), Figure 5.11, p 216 (metabolic genes) and Figure 5.12, p 217 (thermogenic and developmental genes). The results by sex are shown in Appendix D, Table D-10, p 296 (sternal male), Table D-11, p 297 (sternal female), Table D-12, p 298 (subcutaneous male) and Table D-13, p 299 (subcutaneous female).

In some cases (four genes for sternal adipose tissue, two for subcutaneous), data for one of the two groups contained an apparent outlier. Results are presented without removal of outliers, as there was no valid technical reason to exclude them, and they are considered

Table 5.8 Gene expression in sternal adipose tissue by prenatal feeding group. Feeding groups are: undernutrition (U: n = 10; 5M, 5F); 'normal' nutrition (N: n = 6; 6M, 0F); overnutrition (O: n = 10; 4M, 6F). M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (N) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. All groups were compared by Kruskal-Wallis test (p > 0.1 in all cases). Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		
		U	N	O
Adipogenesis	C/EBPα	0.75 ± 0.13	1.00 ± 0.17	1.12 ± 0.20
	NR3C1	0.99 ± 0.13	1.00 ± 0.14	0.91 ± 0.08
	PPARγ	0.84 ± 0.11	1.00 ± 0.16	0.89 ± 0.06
	SREBF1	1.10 ± 0.17	1.00 ± 0.22	1.22 ± 0.25
Metabolism	ADIPOQ	0.78 ± 0.11	1.00 ± 0.13	0.99 ± 0.13
	FABP4	0.83 ± 0.07	1.00 ± 0.09	0.99 ± 0.11
	GPR120	0.63 ± 0.10	1.00 ± 0.16	0.70 ± 0.10
	INSR	0.80 ± 0.12	1.00 ± 0.19	0.93 ± 0.11
	LEP	1.08 ± 0.13	1.00 ± 0.12	0.95 ± 0.11
	RIP140	1.06 ± 0.11	1.00 ± 0.13	0.79 ± 0.08
Thermogenesis	ATF2	1.05 ± 0.09	1.00 ± 0.07	0.95 ± 0.07
	CIDEA	1.07 ± 0.31	1.00 ± 0.27	1.08 ± 0.19
	DIO2	Not quantifiable		
	PGC1α	1.73 ± 0.58	1.00 ± 0.31	0.73 ± 0.20
	PRLR	Not quantifiable		
	UCP1	Not quantifiable		
Development	HOXC9	0.78 ± 0.12	1.00 ± 0.11	0.95 ± 0.09
	LHX8	Not quantifiable		
	PRDM16	Not quantifiable		
	SHOX2	1.21 ± 0.16	1.00 ± 0.10	1.17 ± 0.19

to be genuine biological outliers. However, for completeness, the effect of their removal on mean relative expression and p-values is shown in Table 5.10, p 218, and the effect by sex is shown in Appendix D, Table D-14, p 300 (male) and Table D-15, p 300 (female). In all but one case, removal of the outlier increased the observed effect. Where material, the effect of any outlier removal is also discussed in the following paragraphs.

The results for the two tissues were very similar, with the majority of adipogenic and metabolic genes being significantly downregulated in the HCHF group by around 50 %. The exceptions were FABP4, GPR120, leptin (LEP) and RIP140. FABP4 showed weak evidence of slightly reduced expression in the HCHF group in both tissues (p = 0.811 and p = 0.0868 for sternal and subcutaneous respectively), which became significant when the outliers were removed (p = 0.0257 in both cases). There was no significant difference in the expression of GPR120 between groups in either tissue. Leptin and RIP140 also showed no significant

Table 5.9 Gene expression in subcutaneous adipose tissue by prenatal feeding group. Feeding groups are: undernutrition (U: n = 9; 4M, 5F); 'normal' nutrition (N: n = 6; 6M, 0F); overnutrition (O: n = 9/10; 4M, 5/6F). M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (N) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. All groups were compared by Kruskal-Wallis test (p > 0.1 in all cases). Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		
		U	N	O
Adipogenesis	C/EBPα	0.80 ± 0.11	1.00 ± 0.13	0.92 ± 0.13
	NR3C1	1.01 ± 0.10	1.00 ± 0.13	0.98 ± 0.09
	PPARγ	0.93 ± 0.12	1.00 ± 0.13	1.03 ± 0.14
	SREBF1	0.91 ± 0.08	1.00 ± 0.12	0.97 ± 0.07
Metabolism	ADIPOQ	0.92 ± 0.12	1.00 ± 0.11	1.06 ± 0.14
	FABP4	0.79 ± 0.09	1.00 ± 0.12	0.99 ± 0.13
	GPR120	1.13 ± 0.23	1.00 ± 0.34	1.13 ± 0.22
	INSR	0.77 ± 0.11	1.00 ± 0.16	1.05 ± 0.20
	LEP	0.87 ± 0.13	1.00 ± 0.31	1.02 ± 0.19
	RIP140	0.88 ± 0.14	1.00 ± 0.15	0.89 ± 0.13
Thermogenesis	ATF2	1.01 ± 0.08	1.00 ± 0.09	1.07 ± 0.07
	CIDEA	0.94 ± 0.14	1.00 ± 0.27	1.19 ± 0.27
	DIO2	Not quantifiable		
	PGC1α	2.19 ± 1.20	1.00 ± 0.48	0.93 ± 0.28
	PRLR	Not quantifiable		
	UCP1	Not quantifiable		
Development	HOXC9	0.93 ± 0.14	1.00 ± 0.23	1.02 ± 0.15
	LHX8	Not quantifiable		
	PRDM16	Not quantifiable		
	SHOX2	0.80 ± 0.16	1.00 ± 0.22	0.80 ± 0.17

difference between groups in sternal adipose tissue, but in subcutaneous adipose tissue leptin was significantly upregulated (by around 100 %) and RIP140 was significantly down-regulated (by around 45 %) in the HCHF group.

Of the genes classified as thermogenic or developmental, CIDEA and SHOX2 were also significantly downregulated by around 50 % in the HCHF group in both tissues. PGC1α showed weak evidence of reduced expression in the HCHF group in sternal adipose tissue (around 15 %; p = 0.0723), which further reduced (to around 50 %) and attained significance when the outlier was removed (p = 0.0220). In subcutaneous adipose tissue, there was a significant reduction of PGC1α expression in the HCHF group of around 80 %. ATF2 and HOXC9 showed no significant difference between groups in sternal adipose tissue, but in subcutaneous adipose tissue were significantly downregulated (by around 20 % and 40 % respectively) in the HCHF group.
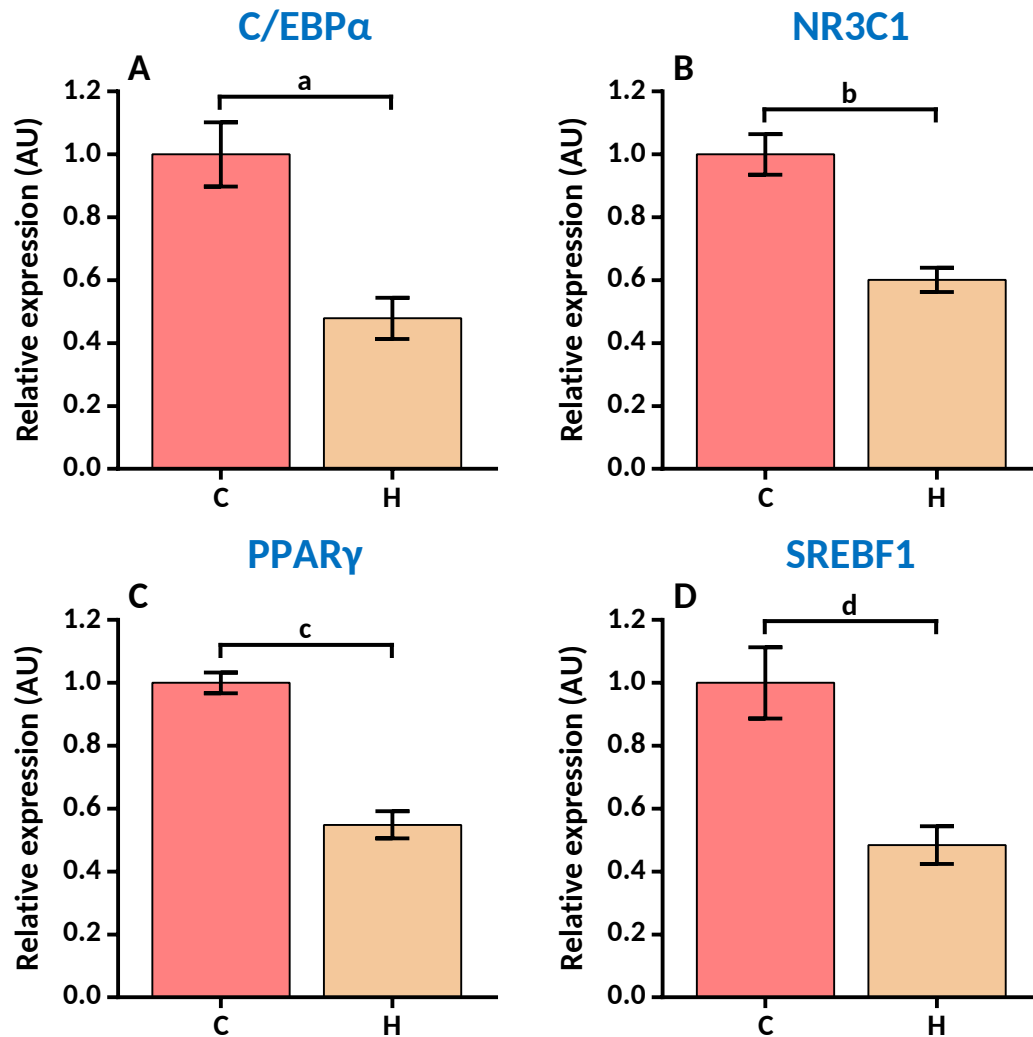
Figure 5.7 Expression of adipogenic genes in sternal adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF) (H: n = 13; 8M, 5F); M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (a: p = 0.0004; b: p < 0.0001; c: p < 0.0001; d: p = 0.0008).

As noted above, the overall effect was reflected in the individual sexes in most cases. However, there were some minor differences. In sternal adipose tissue, males followed the overall pattern of FABP4 expression (weak evidence of slightly reduced expression in the HCHF group), but in females there was no such evidence, even after removal of the outlier. Numerically, the overall reduction in expression of CIDEA in the HCHF group was reflected in both sexes, but was less pronounced and was not significant in the females; however, when the (female) outlier was removed, the difference increased such that there was weak evidence of reduced expression (p = 0.0667).
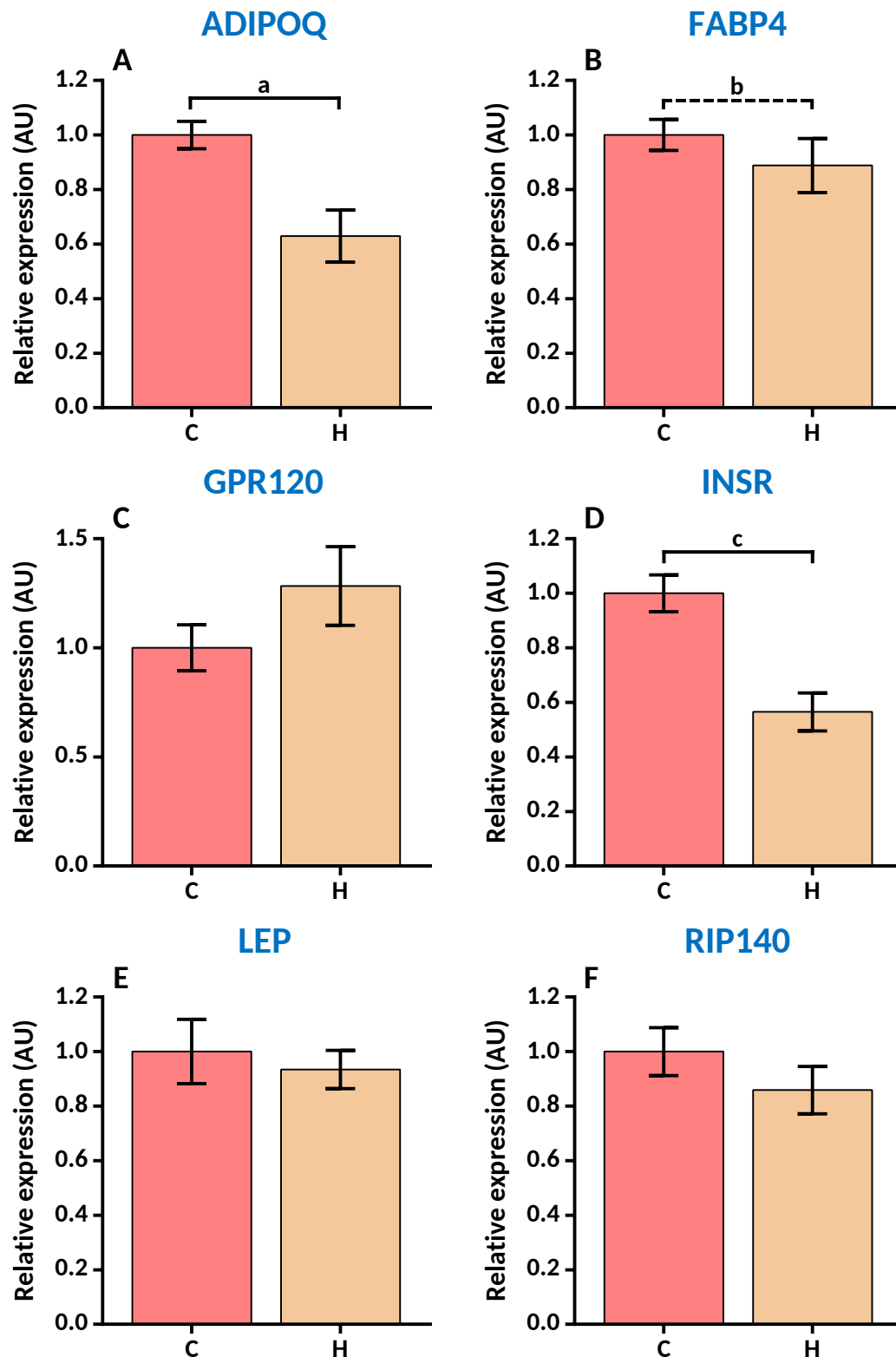
Figure 5.8 Expression of metabolic genes in sternal adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF) (H: n = 13; 8M, 5F); M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (a: p = 0.0006; b: p = 0.0811; c: p = 0.0002; p > 0.1 in all other cases).
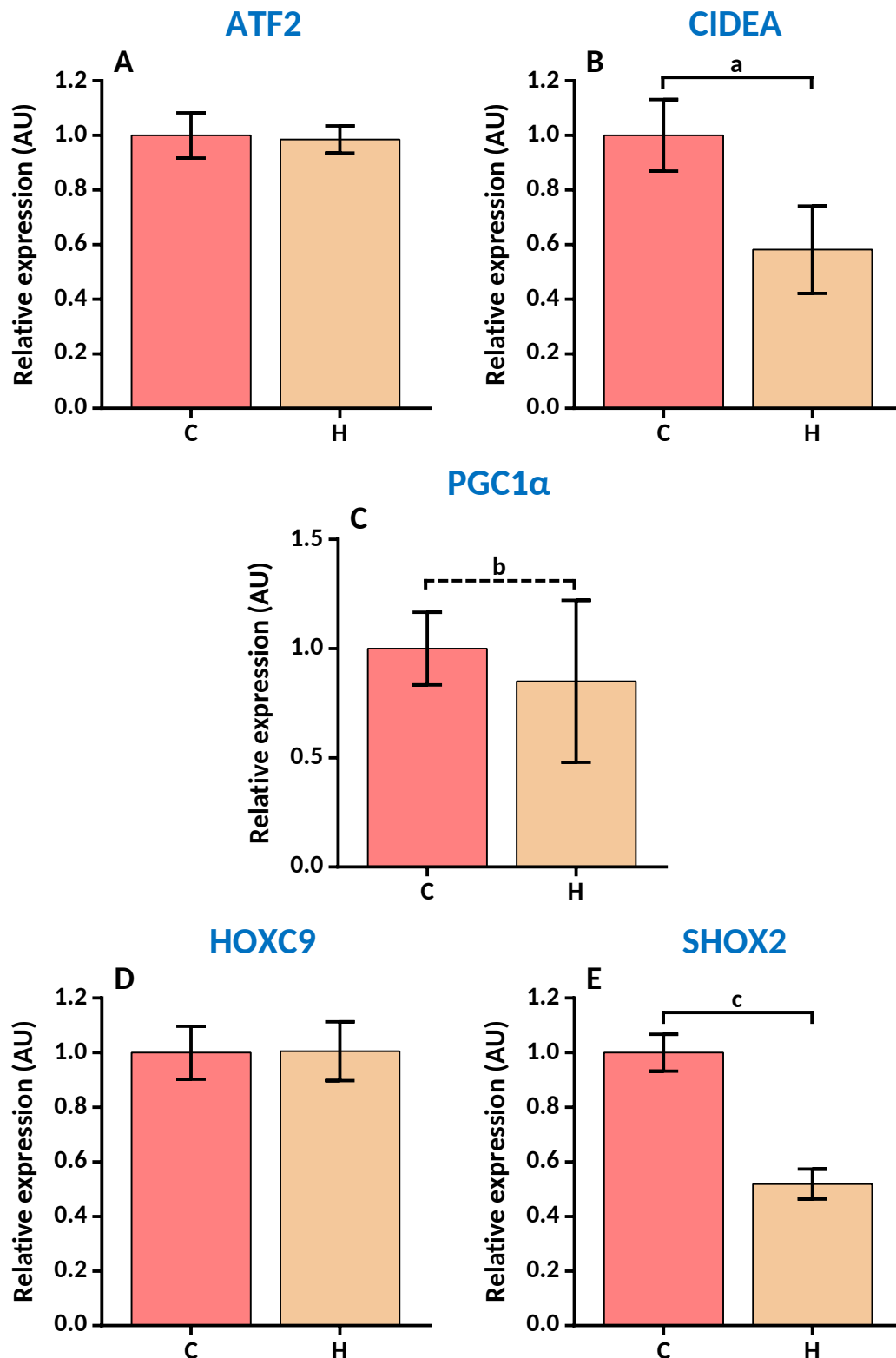
Figure 5.9 Expression of thermogenic and developmental genes in sternal adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF) (H: n = 13; 8M, 5F); M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (a: p = 0.0051; b: p = 0.0723; c: p < 0.0001; p > 0.1 in all other cases). Expression of DIO2, PRLR, UCP1, LHX8 and PRDM16 was barely detectable and not quantifiable.
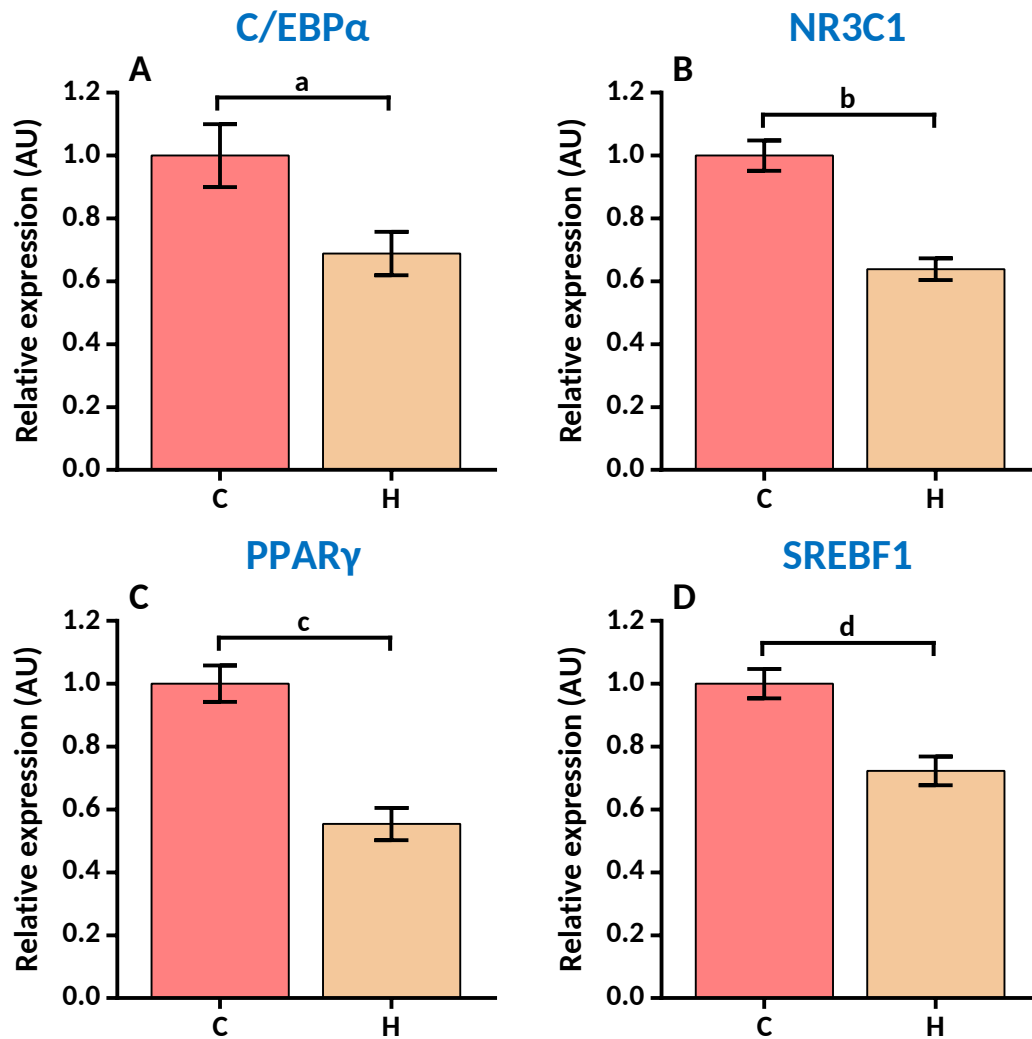
Figure 5.10 Expression of adipogenic genes in subcutaneous adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 12/13; 7M, 5/6F); high-carbohydrate, high-fat (HCHF) (H: n = 12; 7M, 5F); M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (a: p = 0.0284; b: p < 0.0001; c: p < 0.0001; d: p = 0.0005).

In subcutaneous adipose tissue, the overall reduction in C/EBPα expression in the HCHF group of around 30 % was mirrored in the males (albeit with higher significance), but the reduction in the females was smaller and was not significant. The slight overall reduction in FABP4 expression in the HCHF group (only supported by weak evidence before removal of the outlier) was reflected numerically in the individual sexes, but was unsupported in both males and females; however, it became significant in males on removal of the outlier (p = 0.0350). Leptin was upregulated by around 100 % overall in the HCHF group. In males this was raised to around 170 % and remained significant, whereas in females the increase was only around 30 % and was not significant. The 20 % reduction in expression of ATF2 in the HCHF group was reflected numerically in the individual sexes, but there was only weak
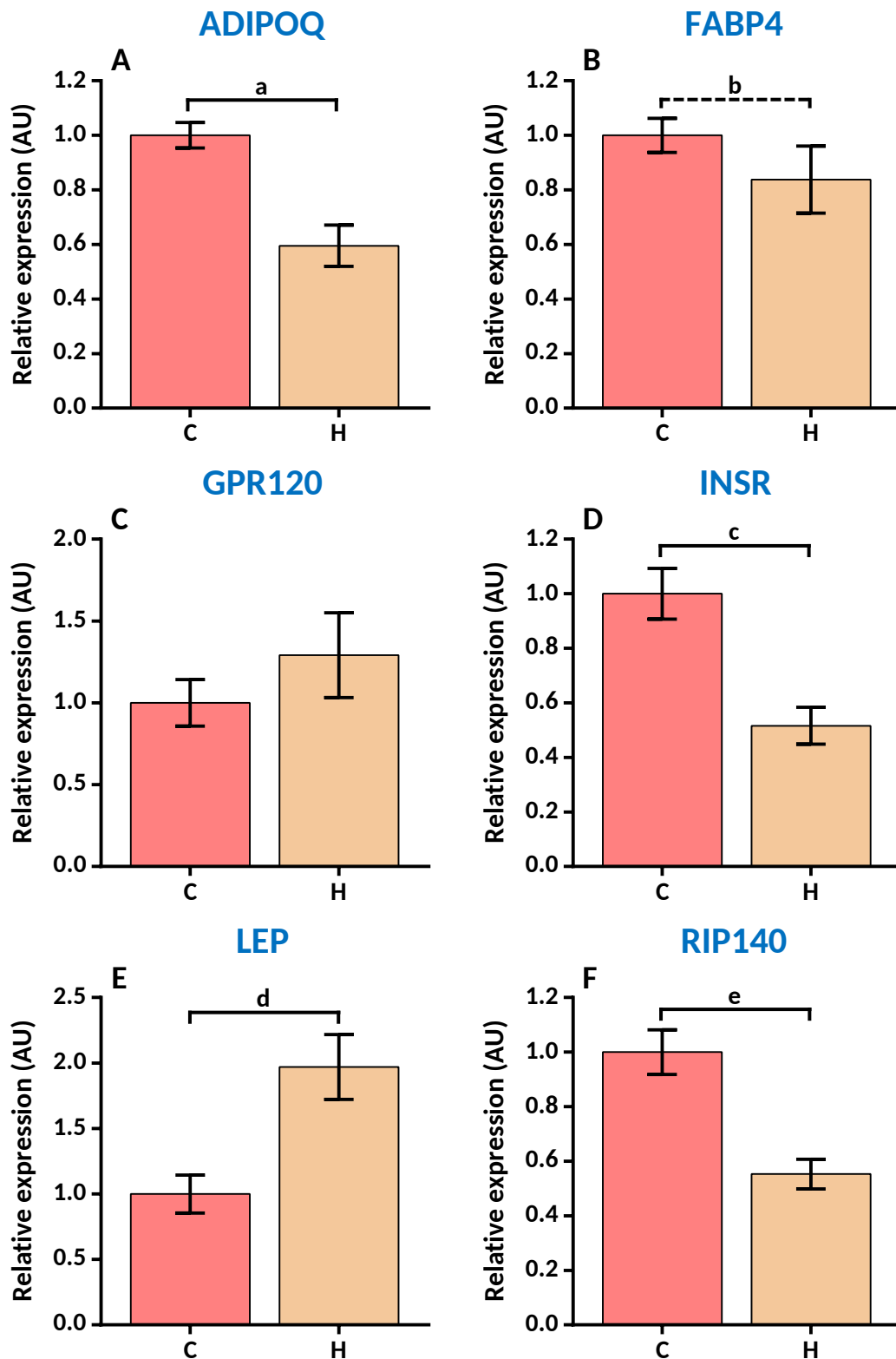
Figure 5.11 Expression of metabolic genes in subcutaneous adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 12/13; 7M, 5/6F); high-carbohydrate, high-fat (HCHF) (H: n = 12; 7M, 5F); M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (a: p = 0.0002; b: p = 0.0868; c: p = 0.0005; d: p = 0.0024; e: p = 0.0002; p > 0.1 in the other case).
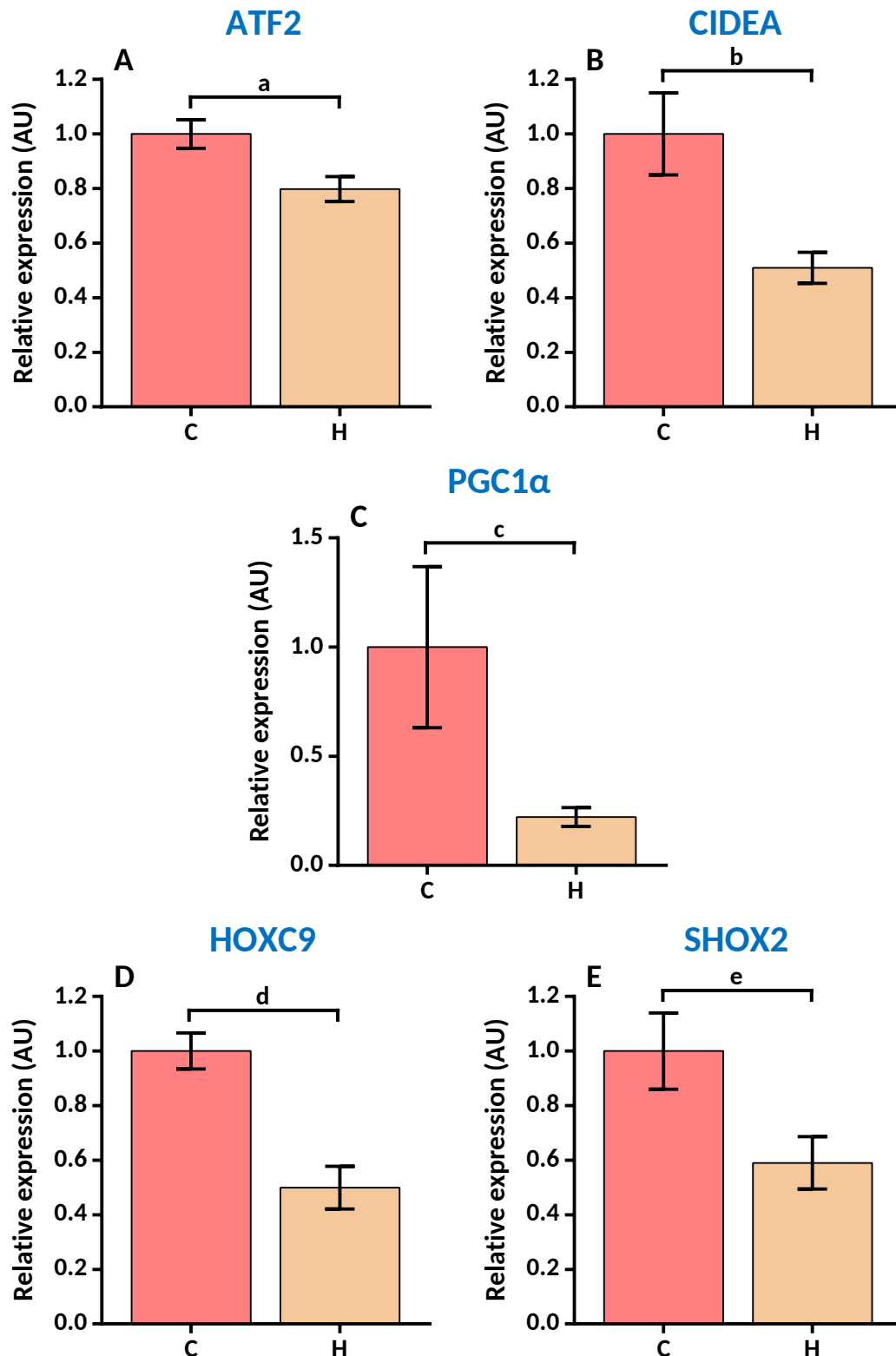
Figure 5.12 Expression of thermogenic and developmental genes in subcutaneous adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 12/13; 7M, 5/6F); high-carbohydrate, high-fat (HCHF) (H: n = 12; 7M, 5F); M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (a: p = 0.0068; b: p = 0.0160; c: p = 0.0080; d: p = 0.0001; e: p = 0.0387). Expression of DIO2, PRLR, UCP1, LHX8 and PRDM16 was barely detectable and not quantifiable.

Table 5.10 Gene expression by postnatal feeding group: effect of outlier removal. Feeding groups are: control (C: n = 12/13; 7M, 5/6F); high-carbohydrate, high-fat (HCHF) (H: n = 12; 7M, 5F); n is shown before removal of the outlier; M is male, F is female. The entries in the Outl(ier) column marked with a superscript 'a' are the same animal. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ (sternal) or PGK1, SDHA, TBP and YWHAZ (subcutaneous). The two groups were compared by Mann-Whitney U-test.

Tissue	Gene	Outl	Complete Rel expression (AU)		p	Minus outlier Rel expression (AU)		p
			C	H		C	H	
Sternal	ADIPOQ	HF [a]	1.00 ± 0.05	0.63 ± 0.10	0.0006	1.00 ± 0.05	0.54 ± 0.04	< 0.0001
	FABP4	HF [a]	1.00 ± 0.06	0.89 ± 0.10	0.0811	1.00 ± 0.06	0.81 ± 0.06	0.0257
	CIDEA	HF	1.00 ± 0.13	0.58 ± 0.16	0.0051	1.00 ± 0.13	0.43 ± 0.07	0.0006
	PGC1α	HM	1.00 ± 0.17	0.85 ± 0.37	0.0723	1.00 ± 0.17	0.50 ± 0.13	0.0220
Subcutaneous	FABP4	HM	1.00 ± 0.06	0.84 ± 0.12	0.0868	1.00 ± 0.06	0.73 ± 0.07	0.0257
	PGC1α	CM	1.00 ± 0.37	0.22 ± 0.04	0.0080	1.00 ± 0.26	0.33 ± 0.07	0.0145

evidence for a difference (p = 0.0728 and p = 0.0952 respectively for males and females). The overall reduction in CIDEA expression in the HCHF group of around 50 % was reflected numerically in both the individual sexes, but there was only weak evidence for a difference in males (p = 0.0728) and it was not significant in females. There was an overall reduction in PGC1α expression in the control group of around 80 %, which decreased to around 65 % (with a lower level of significance) on removal of a very large outlier from that group. Both sexes also showed a similar large reduction in expression in the HCHF group, which was significant in males and supported by weak evidence in females (p = 0.0823). However, removal of the outlier decreased the effect in males to around 55 % and significance was lost, although the effect was supported by weak evidence (p = 0.0734). Finally, the overall reduction in SHOX2 expression in the HCHF group of around 40 % was increased in males (to around 50 %) and remained significant, but lower in females (around 25 %) and was not significant.

5.4.3 Statistical power

The effect size for differences in relative tissue weights between the postnatal HCHF and control groups was so high (around 4.9) that power was calculated to be 100 %. The effect size for differences in gene expression was lower (around 1.0), and power was calculated to be around 66 %. In order to increase the power to the conventionally accepted level of 80 % for the same effect size, calculations indicated that the sample size for each group would need to be increased from 13 to 20 (ie a total of 40 animals).

5.4.4 Summaries of results

Summaries of results for this chapter are shown in Table 5.11, below (prenatal) and Table 5.12, below (postnatal).

Table 5.11 Summary of results by prenatal group. Differences are shown for feeding group within sex (first six columns) and sex within feeding group (last three columns). An up arrow (↑⇧) indicates that body weight is higher than the reference group, a down arrow (↓) indicates that body weight is lower than the reference group and a horizontal dash (–) indicates that there is no difference in body weight between the two groups (p > 0.1). An outline arrow (⇧) indicates weak evidence of a change (ie 0.05 ≤ p < 0.1).

Item	U or O relative to N				U relative to O		M relative to F		
	Male		Female		M	F	U	N	O
	U	O	U	O					
Birth weight	↓	–	–	–	↓	–	–	–	↑
Body weight at 6 months	–	–	–	–	–	–	–	↑	⇧

Table 5.12 Summary of results by postnatal group. Tissues are mesenteric (Mes), perirenal (Per), sternal (Ste) and subcutaneous (Sub). An up arrow (↑) indicates that tissue weight or gene expression is higher in the high-fat, high-carbohydrate (HCHF) group than in controls, a down arrow (↓⇩) indicates that gene expression is lower in the HCHF group than in controls, and a horizontal dash (–) indicates no difference in gene expression between the HCHF group and controls (p > 0.1). An outline arrow (⇩) indicates weak evidence of a change (ie 0.05 ≤ p < 0.1).

Item	Category	Gene	Body	Tissue			
				Mes	Per	Ste	Sub
Body weight			↑				
Tissue weight	Absolute			↑	↑	↑	↑
	Relative			↑	↑	↑	↑
Gene expression	Adipogenesis	C/EBPα				↓	↓
		NR3C1				↓	↓
		PPARγ				↓	↓
		SREBF1				↓	↓
	Metabolism	ADIPOQ				↓	↓
		FABP4				⇩	⇩
		GPR120				–	–
		INSR				↓	↓
		LEP				–	↑
		RIP140				–	↓
	Thermogenesis	ATF2				–	↓
		CIDEA				↓	↓
		PGC1α				⇩	↓
	Development	HOXC9				–	↓
		SHOX2				↓	↓

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5.5 Discussion

5.5.1 Body and tissue weights

5.5.1.1 Maternal undernutrition in late gestation reduces offspring birth weight in males only

Male lambs whose mothers were undernourished in late gestation weighed less at birth by some 17–21 % than those whose mothers were normally fed or overnourished. In contrast, differences in maternal nutrition did not affect the birth weight of female lambs. While a relationship between late gestational undernutrition and reduced birth weight is well supported, at least in sheep [Kenyon & Blair, 2014], there is not much information in the literature about sex-specific effects. One study, however, has reported a sex-specific, but opposite, effect in sheep, whereby the female offspring of undernourished mothers had a lower birth weight than controls, while no significant difference was seen in the males. It is not directly comparable, though, as the breed was different, the lambs were singletons, the period of undernourishment was only 20 days from day 105 of term, the control group was fed *ad libitum* (up to 15 MJ/d) for that period, and the food restriction was severe (only 0.3–0.5 MJ/d) [Oliver *et al*, 2002]. Another study demonstrated a sex-specific effect of parenteral L-arginine administration from day 100 of gestation to term on the offspring birth weight of well-fed twin-bearing ewes. In this case the female arginine-supplemented lambs were some 12 % heavier than controls, whereas the birth weights of male lambs did not significantly differ between the two groups [McCoard *et al*, 2013]. No reports of any sex-specific effects of nutrition on birth weight in humans could be found in the literature, possibly because they have not been addressed, but there is more general evidence that the human maternal environment can have a sex-specific effect on birth weight, in that it has been found that heavy smoking by pregnant mothers has a greater negative effect on the birth weight of girls than boys [Voigt *et al*, 2006]. In any event, the possibility that nutritional and other interventions during gestation can reduce birth weight in a sex-specific manner is potentially of interest, because low birth weight has been linked with abdominal fat deposition [Law *et al*, 1992] and conditions such as high blood pressure [Law & Shiell, 1996], T2DM [Whincup *et al*, 2008], insulin resistance [Newsome *et al*, 2003] and osteoporosis [Martínez-Mesa *et al*, 2013] in adulthood. It would be interesting to see if this apparent maintenance of normal birth weight in the female progeny of undernourished mothers is replicated in future large animal and human studies, and if so, to determine whether it correlates with an improved prognosis in adulthood, to what extent the timing and degree of restriction is a factor, and what the possible mechanisms might be for the

protective effect in females. Finally, the fact that there were no significant differences in the mean body weights of males at 6 months of age between the three groups implies that the low birth weight male offspring of undernourished mothers grew at an accelerated rate relative to their peers, which is consistent with the well-established phenomenon of 'catch-up' growth [Boersma & Wit, 1997; Prader *et al*, 1963].

5.5.1.2 An obesogenic diet substantially increases adipose tissue weight in all depots measured, with the greatest effect in the perirenal depot

Relative to body weight, lambs on the obesogenic HCHF diet had some 4½- to 5-fold more adipose tissue mass at six months of age in their mesenteric, sternal and subcutaneous fat depots than their conventionally-fed counterparts. In contrast, the rate of fat deposition in the perirenal depot of HCHF lambs was nearly double that of the other depots measured, with HCHF diet lambs having some 9-fold more perirenal adipose tissue mass than animals on the conventional diet.[38] The implications of this preferential targeting of the perirenal fat depot have already been discussed by Khanal *et al* [2014], so are not addressed here, other than to note that abdominal adiposity, which is reflected in upper-body obesity and a high WHR in humans, has been linked with an increased risk of CVD, T2DM, certain cancers, and a variety of other diseases and conditions [Montague & O'Rahilly, 2000].

5.5.1.3 Females fed on an obesogenic diet have more perirenal adipose tissue relative to body weight than males after six months

The finding that female lambs on an obesogenic diet had more perirenal adipose tissue relative to body weight than their male counterparts (by around 36 %) is a little surprising. In humans, females have a greater percentage of body fat than males, but males have a higher percentage of visceral/abdominal fat [Blaak, 2001; Lemieux *et al*, 1993], something which is reflected in the stereotypical male 'apple' shape and female 'pear' shape (termed android and gynoid obesity respectively). Moreover, it has been specifically reported that human males have more perirenal fat (and less subcutaneous fat) than females [Eisner *et al*, 2010]. Notwithstanding inter-species differences, therefore, it might be expected that male lambs on an obesogenic diet would deposit more perirenal fat than females. Although the contrary finding in this study should be treated with a certain amount of caution, given that there are no female offspring of normally-fed mothers in the sampled subset, it is confirmed by the linear model used by Khanal *et al* [2014], which should compensate for the sexual imbalance. Furthermore, there has been a report that 3 week-old female lambs

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[38] There was also a concomitant reduction in kidney size in the HCHF group [Khanal *et al*, 2014].

have more perirenal adipose tissue than males, irrespective of birth weight [Duffield *et al*, 2009], and a more recent report indicating that 11 week-old male lambs have less perirenal fat mass per kg body weight than females [Wallace *et al*, 2014], so there is some precedent for this apparent disparity between humans and sheep.

5.5.2 Gene expression in sternal and subcutaneous adipose tissue

Colleagues in Denmark have carried out (currently unpublished) work on the expression of adipogenic, metabolic and inflammatory genes in perirenal, mesenteric, epicardial and subcutaneous (but not sternal) adipose tissue from the study, which is referred to in the following paragraphs [Prabhat Khanal, personal communication, 28 February 2014]. There is some overlap between the work reported in this chapter and that done by colleagues on subcutaneous adipose tissue, but the effect is minimal, as the majority of the adipogenic and metabolic genes used were different, and in the event only two genes were duplicated (being PPARγ and FABP4). Other adipogenic and metabolic genes examined by colleagues were C/EBPβ, the angiogenic factor VEGFA, GLUT4, and the lipid metabolism genes fatty acid synthase (FASN), hormone-sensitive lipase (HSL) and lipoprotein lipase. As was the case in the paper by Khanal *et al* [2014], colleagues prepared their statistics using a linear model, with results presented as least square means.

5.5.2.1 Late gestational over- and undernutrition has no effect on the expression of adipogenic, metabolic, thermogenic and developmental genes in the sternal and subcutaneous adipose tissue of offspring at six months of age

It was hypothesised that late gestational over- and undernutrition in ewes would alter the expression of adipogenic, metabolic and thermogenic genes in the adipose tissue of their offspring at six months of age. In the event, no differences in expression were detected between the three prenatal groups for any of the genes tested, including developmental genes. Similarly, colleagues in Denmark found that modifications in prenatal nutrition had no effect on the expression of adipogenic or metabolic genes in perirenal or mesenteric adipose tissue. However, they did find that three genes, FABP4, GLUT4 and PPARγ, were downregulated ($p < 0.05$) in the undernutrition group in epicardial adipose tissue. They also found that HSL was downregulated in the overnutrition ($p < 0.001$) and undernutrition groups ($p < 0.05$), and that FABP4 was downregulated in the undernutrition group ($p < 0.05$) with weak evidence of a similar effect in the overnutrition group ($p < 0.1$), in subcutaneous adipose tissue [P Khanal, personal communication, 28 February 2014]. The result for FABP4 conflicts with that of this study, and it may be that a linear model gives a better indication of the true picture. In any event, there is clearly no pattern to any prenatal effect on gene

expression in adipose tissue, with the observed changes being gene- rather than category-specific. Finally, it may be that the timing of interventions is a factor, as undernutrition seems to have more effect on subsequent adult obesity in the first half of pregnancy, at least in humans [Ravelli *et al*, 1976]. Studies on overnutrition seem to focus on maternal diabetes or obesity, where exposure to excess nutrients is over a more prolonged period, so again it may be that the first half of pregnancy is the critical window for subsequent adult obesity, and consequently any programming of adipose tissue.

5.5.2.2 An obesogenic diet reduces the expression of adipogenic and metabolic genes in sternal and subcutaneous adipose tissue

It was expected that an obesogenic HCHF diet would increase the amount of adipose tissue in six month-old lambs by a combination of hypertrophy and hyperplasia. The latter would entail further differentiation of precursor cells, so it was hypothesised that the expression of adipogenic and metabolic genes in the adipose tissue of lambs on the HCHF diet would increase relative to that of controls. In the event, the opposite was observed, with the expression of most adipogenic and metabolic genes downregulated by some 50 % in both sternal and subcutaneous adipose tissue in the HCHF group compared to the control group. A similar result, albeit with a largely different gene set, was obtained by colleagues in Denmark for subcutaneous adipose tissue, and also for perirenal, mesenteric and epicardial adipose tissue. Conversely, they also found that inflammatory genes were upregulated in the HCHF group in the four adipose tissue depots they tested. Furthermore, their work on histology determined that the HCHF diet induced general hypertrophy and hyperplasia in all four adipose tissues tested [P Khanal, personal communication, 28 February 2014].

While the result that adipogenic and metabolic genes are downregulated in the adipose tissue of obese lambs may seem surprising, the phenomenon has also been observed in mice. Nadler *et al* [2000] used DNA microarrays to study differences in gene expression in the epididymal adipose tissue of 14 week-old mice that were genetically lean, obese or obese-diabetic, and found that genes normally associated with adipocyte differentiation, including SREBF1, PPARγ, and C/EBPα, were downregulated in the obese mice compared to the lean. They concluded that some degree of dedifferentiation had taken place in the adipose tissue of obese animals. Similarly, Moraes *et al* [2003] used a microarray and RT-qPCR to determine changes in gene expression between diet-induced obese (DIO) mice and controls. After eight weeks, the DIO mice were clearly obese, and had reduced expression in abdominal adipose tissue of several genes involved in lipid metabolism (including FASN) and some other genes that are markers of adipocyte differentiation or involved in energy metabolism, although in this case there was no change in the expression of SREBF1. The

authors of this paper also conclude that adipocytes are involved in a dedifferentiation process, possibly as a result of their enlargement by obesity. In contrast, in a similar study on rats, López *et al* [2003] found that the expression of PPARγ, C/EBPα and a number of genes involved in lipid metabolism were upregulated in the epididymal adipose tissue of DIO animals compared to wild types after 65 days on their respective diets. While the results for mice and rats seem to be contradictory, it may well be that they reflect different stages in the development of obesity rather than genuine inter-species differences [Al-Hasani & Joost, 2005], as is discussed in the following paragraph.

In terms of the rationale and potential mechanism for the observed downregulation of adipogenic and metabolic genes in the adipose tissue of obese lambs, the corresponding upregulation of inflammatory genes detected by colleagues in the four adipose tissues they examined may provide a clue, and it would be useful to extend the testing of inflammatory genes to sternal adipose tissue. One such gene in particular that was upregulated in all the tissues they tested was TNFα, although there was only weak evidence (p = 0.072) for the effect in subcutaneous adipose tissue. It has been shown that TNFα is a powerful inhibitor of adipogenesis, and that it can also suppress the expression of adipocyte-specific genes in fully mature adipocytes. In particular, it downregulates the key factors PPARγ and C/EBPα, which are essential for both adipogenesis (Section 1.2.5, p 31; Figure 1.5, p 34) and maintenance of the fully-differentiated adipocyte state [Sethi & Hotamisligil, 1999]. An increase in adipocyte size (ie hypertrophy) is associated with the infiltration of macrophages into adipose tissue, thereby promoting inflammation, insulin resistance and TNFα secretion. Indeed, macrophages are responsible for almost all TNFα expression in adipose tissue [Weisberg *et al*, 2003]. A potential model for the mechanism, therefore, is that at the onset of a high-fat diet adipose tissue grows by a combination of hypertrophy and hyperplasia, the latter leading to an upregulation of adipogenic and metabolic genes. As the size of adipocytes grows through hypertrophy, macrophages infiltrate the tissue and increase secretion of TNFα, thereby suppressing the expression of adipogenic and metabolic genes. This leads to dedifferentiation of adipocytes, a loss of lipogenic capability, and ectopic fat accumulation, particularly in the liver [Al-Hasani & Joost, 2005; Gustafson *et al*, 2009].

5.5.2.3 An obesogenic diet has different effects on the mRNA expression of leptin and RIP140 in sternal and subcutaneous adipose tissue

While an obesogenic diet downregulated most adipogenic and metabolic genes in both sternal and subcutaneous adipose tissue, the effect on expression of leptin (LEP) and RIP140 was tissue-specific. In sternal adipose tissue, there was no change in the expression of either gene in animals on the HCHF diet relative to controls. In subcutaneous adipose

tissue, however, leptin was upregulated by some 100 % in the obese animals, while RIP140 was downregulated by about 45 %.

The upregulation of leptin in the subcutaneous adipose tissue of animals on an HCHF diet is consistent with a recent bovine study which reported that it was upregulated in the subcutaneous (and intramuscular) adipose tissue of 30 month-old steers on a high-energy high-protein diet compared to roughage-fed controls, but not in the perirenal, mesenteric or intermuscular depots [Asano et al, 2013]. However, a case could be made for either an increase or decrease in leptin expression in lambs on the HCHF diet. On the one hand, leptin expression is correlated with fat mass [Ahima & Flier, 2000; Friedman & Halaas, 1998], which increased in the HCHF group in both tissues, and inversely correlated with adiponectin expression [Arita et al, 1999; Stefan et al, 2002], which decreased in the two tissues; on the other hand, dedifferentiation of adipocytes, particularly downregulation of C/EBPα, a transcriptional activator of leptin [Hwang et al, 1997], might be expected to decrease its expression, as preadipocytes do not express leptin [Wang et al, 2008]. It may well be that the balance of factors (eg adipocyte size, ratio of hypertrophy to hyperplasia) determining these two possible outcomes varies between tissues, resulting in the tissue-specific difference in leptin expression seen in obese animals. An interesting possibility is that TNFα is again a relevant factor, although there is some conflict in the literature as to its effect on leptin expression and secretion, which has been described as "complex" [Fawcett et al, 2000]. Some reports indicate that it induces leptin expression and secretion in adipose tissue [Finck & Johnson, 2000; Sarraf et al, 1997], others that it inhibits it [Alexander et al, 2011; Fawcett et al, 2000], and yet others that suggest a dual effect, with expression being inhibited and secretion being stimulated [Kirchgessner et al, 1997; Zhang et al, 2000]. However, in this study TNFα expression has not yet been tested in sternal adipose tissue, and colleagues have not tested leptin expression in perirenal, mesenteric and epicardial adipose tissue, so further work would be needed to ascertain whether there is any correlation between the two.

One further consideration in relation to leptin is that the increase in its expression in lambs on the HCHF diet relative to controls in subcutaneous adipose tissue was highly sex-specific, with a significant increase of some 170 % in males, but no significant difference in females (although there was a numerical increase in mean expression of around 30 %). It is well-established that there is sexual dimorphism in circulating leptin levels in a number of species, with the male to female ratio of plasma leptin concentration remaining relatively constant irrespective of BMI. In humans and sheep, females have higher levels [Blache et al, 2000; Hellström et al, 2000], while in rats the opposite effect has been reported [Landt et al, 1998]. It would not be unexpected, therefore, for there to be similar dimorphism in leptin

mRNA expression. However, the fact that the effect is irrespective of BMI implies that any percentage changes in expression in the two sexes should be similar, which is contrary to what was observed. Furthermore, one human study has reported a positive relationship between BMI and leptin mRNA expression in the subcutaneous adipose tissue of women [Montague *et al*, 1997], while another (on women only) showed increased leptin (and TNFα) expression in the subcutaneous adipose tissue of obese females compared to lean controls [Bulló *et al*, 2002]. The findings of this study therefore seem to conflict with what has been reported previously, particularly in humans, and further work will be required to ascertain whether the effect is genuine or anomalous. Finally, colleagues in Denmark have reported that the plasma leptin concentration of lambs on the HCHF diet was about twice that of those on the control diet, although no analysis was done by sex [Khanal *et al*, 2014]. This is consistent with the two-fold increase in leptin expression in the subcutaneous (but not sternal) adipose tissue of HCHF lambs.

Mice deficient in RIP140 are lean and resistant to diet-induced obesity [Leonardsson *et al*, 2004], so it might be expected that RIP140 expression would increase with obesity. In contrast, a human study has reported that morbidly obese subjects expressed lower RIP140 mRNA and protein in omental adipose tissue than lean controls [Catalán *et al*, 2009]. It is likely that in mice the anti-obesogenic effect of RIP140 ablation is caused by the removal of its inhibitory effect on thermogenesis, with the resulting increased energy expenditure accounting for the lean phenotype [Christian *et al*, 2005]. In humans and sheep, a reduction of the inhibition on a less well-developed thermogenic capability may be insufficient to counteract other factors that promote an obese phenotype. In any event, the reduction in RIP140 expression in the subcutaneous adipose tissue of lambs on the HCHF diet relative to controls is consistent with the latter (human) study. It is also consistent with what might be expected from dedifferentiation, as there is a progressive increase in RIP140 expression during the course of adipogenesis [Soukas *et al*, 2001]. However, while there is some basis and support for the reduction in RIP140 expression in the subcutaneous adipose tissue of obese lambs, it is not evident why its expression is unchanged in sternal adipose tissue.

5.5.2.4 Expression of thermogenic genes in the sternal and subcutaneous adipose tissue of six month-old lambs is barely detectable, and the effect of an HCHF diet on such expression is inconclusive

With the exception of ATF2, all genes classified as thermogenic were expressed at very low levels in both sternal and subcutaneous adipose tissue (Table 5.7, p 209). ATF2 is a multi-functional gene (Section 1.8.4.1, p 59) [Bhoumik *et al*, 2005; Bruhat *et al*, 2007; Lee *et al*, 2001] that was expressed at similar levels to most of the adipogenic and metabolic genes tested,

so it is likely that its role in thermogenesis (ie promotion of UCP1 and PGC1α expression (Figure 1.3, p 26) [Cao *et al*, 2004; Collins *et al*, 2010]) is subordinate to some other function, as suggested in the sternal adipose tissue ontogeny study (Section 3.5.2.2, p 142). It has a role in early stage adipogenesis [Lee *et al*, 2001; Maekawa *et al*, 2010], promotes the expression of proinflammatory genes, including TNFα, and is highly expressed in infiltrating macrophages [Brinkman *et al*, 1999; Yu *et al*, 2014]. Its expression was reduced by around 20 % in the subcutaneous adipose tissue of lambs on the HCHF diet compared to controls, but there was no difference between groups in sternal adipose tissue. Both these results are perhaps a little surprising, as its expression might have been expected to increase in line with that of inflammatory genes in the adipose tissue of obese lambs. Although other adipogenic genes were downregulated in the HCHF group, any dedifferentiation of adipocytes could be expected to have less effect on the expression of genes involved in the early part of the differentiation process. However, further work will be required to unravel the competing influences of thermogenesis, adipogenesis and inflammation on its expression.

The gene for the lipid-droplet protein CIDEA was originally classified as thermogenic, as it is highly expressed in the BAT of mice, but not expressed in murine WAT (Section 1.8.4.2, p 60) [Zhou *et al*, 2003]. However, it is highly expressed in human WAT [Nordström *et al*, 2005], and the ontogeny study suggests that it does not play a key role in thermogenesis in sheep, at least in sternal adipose tissue (Section 3.5.2.2, p 142). Its expression is relatively low, being at the top end of the range for the definitive thermogenic genes, but well below that of adipogenic and metabolic genes. Its expression was reduced by around 50 % in both tissues in lambs on an HCHF diet compared to controls, which is in line with the findings for most adipogenic and metabolic genes. It is also consistent with a report that its expression is reduced twofold (ie by 50 %) in the subcutaneous adipose tissue of obese humans [Nordström *et al*, 2005]. The same report also finds that its expression is reduced by TNFα treatment, which may provide an explanation for the effect. However, Asano *et al* [2013], in their bovine study, found no significant difference in CIDEA expression between the high-energy, high-protein group and roughage-fed controls in the subcutaneous and mesenteric adipose tissue of 30 month-old steers. Mean expression was actually higher numerically in the former group, but their sample sizes were small (four animals in each group). These diverse findings highlight the fact that CIDEA expression is unusually species-specific, and further work will be required to fully understand its role in sheep.

The remaining genes that were originally classified as thermogenic, UCP1, PGC1α, DIO2 and PRLR, are considered together with PRDM16, which was grouped with developmental genes as it determines the BAT fate of adipomyocite precursors. However, it also seems to have a role in the 'browning' of subcutaneous WAT and the initiation of thermogenesis in

that tissue (Section 1.8.5.3, p 64) [Seale *et al,* 2011]. DIO2, PRLR, UCP1 and PRDM16 were all expressed at levels that were too low for reliable quantification in either tissue. PGC1α, which was also expressed at very low, albeit quantifiable, levels, was significantly down-regulated in the HCHF group in subcutaneous adipose tissue, and there was weak evidence of reduced expression in that group in sternal adipose tissue. This conflicts with the stated hypothesis that a high-fat diet would increase the expression of thermogenic genes in adipose tissue, and also with the results of Asano *et al* [2013], who reported that PGC1α was more highly expressed in the high-energy, high-protein group compared to roughage-fed controls in the subcutaneous adipose tissue of 30 month-old steers, though the difference was not significant. However, they found that UCP1, DIO2, PRDM16 and two other thermogenic genes, cytochrome c oxidase (COX) 1 and COX8B, had significantly higher expression in the high-energy, high-protein group than controls in subcutaneous, but not mesenteric, adipose tissue.

It is hard to draw firm conclusions from the results of this study given that expression of four of the five definitive thermogenic genes could not be reliably quantified. However, the expression of thermogenic genes in the sternal and subcutaneous adipose tissue of six month-old lambs is so low that any changes in expression of less than an order of magnitude are unlikely to have important biological consequences.

5.5.2.5 An obesogenic diet reduces the expression of certain developmental genes in sternal and/or subcutaneous adipose tissue

A few developmental genes were originally included in the study as they had been labelled markers of brite/beige adipose tissue or classical BAT [Waldén *et al,* 2012]. Although the ontogeny study on sternal adipose tissue (Section 3.5.2.3, p 143) and other recent evidence [de Jong *et al,* 2015] indicates they are not informative as to adipose tissue type, they are still potentially of interest because they are differentially expressed in the various adipose tissue depots of both humans and mice [Gesta *et al,* 2006; Yamamoto *et al,* 2010]. Expression of LHX8, however, was too low to be reliably quantified in either sternal or subcutaneous adipose tissue.

Expression of HOXC9 (Section 1.8.5.1, p 63) was much higher than that of thermogenic and other developmental genes, being at the bottom end of the range for adipogenic and metabolic genes. Like most adipogenic and metabolic genes, it was downregulated by some 50 % in the subcutaneous adipose tissue of lambs on the HCHF diet compared to controls, but there was no difference in expression between the two groups in sternal adipose tissue. Its role (and that of other HOX genes) in adipose tissue is not clear [Yamamoto *et al,* 2010], but its relatively high level of expression would suggest that it is not insignificant,

and its expression has been reported to increase when WAT is subjected to 'browning' [Petrovic *et al*, 2010]. It is not clear why an obesogenic diet should alter its expression in subcutaneous, but not sternal, adipose tissue, and further work will be needed to ascertain its role, and understand its expression pattern, in the different adipose depots.

SHOX2 (Section 1.8.5.4, p 65) was expressed at a level similar to the top end of the range for thermogenic genes, and seemed to be more highly expressed in subcutaneous than sternal adipose tissue (by about 2½-fold; Table 5.7, p 209).[39] This would be consistent with a report that it is more highly expressed in subcutaneous than visceral adipose tissue [Lee *et al*, 2013], if sternal adipose tissue is considered more visceral than subcutaneous. Like most adipogenic and metabolic genes, it was downregulated by around 50 % in both tissues of lambs on the HCHF diet compared to those of controls (in contrast to HOXC9 which was only downregulated in subcutaneous adipose tissue). This is perhaps surprising, as in mice and humans its expression does not change during adipogenesis [Lee *et al*, 2013], so it would not be expected to change on dedifferentiation. It seems to play a role in humans and mice in determining the function and distribution of adipose tissue, possibly through regulation of ADRB3 [Lee *et al*, 2013]. It has been found that its expression is higher in the subcutaneous adipose tissue of humans with visceral adiposity than in lean individuals or those with subcutaneous adiposity, and that in male mice its disruption in adipose tissue protects against diet-induced obesity [Lee *et al*, 2013]. This seems to conflict with the results of this study, where it is downregulated in the (viscerally obese) lambs on the HCHF diet. Further work will be required, therefore, to ascertain its role in ovine adipose tissue.

5.6 Summary and conclusions

A nutritional study was carried out on sheep in which twin-pregnant ewes were allocated to one of three feeding groups in late gestation, undernutrition, 'normal' nutrition or overnutrition, and their offspring were each allocated to one of two diets, conventional roughage or HCHF. A subset of the offspring was sampled at six months of age. It was hypothesised that late gestational over- and undernutrition would change the expression, and that a high-fat postnatal diet would increase the expression, of adipogenic, metabolic and thermogenic genes in the adipose tissue of 6 month-old sheep.

It was found that male, but not female offspring of undernourished mothers had lower birth weights than offspring of normally fed or overnourished mothers, but that the body weight of these animals had 'caught up' to that of their peers by six months of age. No information was found in the literature about sex-specific effects of nutrition on birth

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[39] This is only an estimate, based on mean C_T values, as relative quantification was used in this study.

weight in humans, and the only study in sheep reporting such an effect found a higher birth weight in the female offspring of undernourished mothers, though there were substantial differences in the experimental protocol. It was found that animals on the HCHF diet had substantially more adipose tissue in all the depots measured, but that deposition in the perirenal depot was at about twice the rate of the other depots, indicating a trend towards visceral adiposity. Somewhat surprisingly, females had more perirenal fat relative to body weight than males at six months of age, an effect which has also been reported in another ovine study, but in that case on much younger (three week-old) lambs.

Contrary to what was hypothesised, late gestational over- and undernutrition had no effect on gene expression in either of the two tissues tested. In contrast, an obesogenic diet downregulated the expression of most adipogenic and metabolic genes by around 50 % in the sternal and subcutaneous adipose tissue of 6 month-old lambs. It was hypothesised, however, that there would be an increase in expression of these genes as a result of hyperplasia. The opposite effect may be the result of dedifferentiation of adipocytes as a result of hypertrophy, subsequent macrophage infiltration and upregulation of proinflammatory genes, including TNFα, but further work will be required to support this hypothesis. One exception to the general downward trend was leptin, expression of which was unchanged by a high-fat diet in sternal adipose tissue, but which was increased in the subcutaneous adipose tissue of males in a highly sex-specific manner. This finding conflicts with some previous reports that indicate that leptin expression in subcutaneous adipose tissue is correlated with BMI in females, and further work will be required to ascertain whether it is replicable or anomalous. Expression of thermogenic genes was extremely low in both tissues and mainly unquantifiable. The effect of an obesogenic diet on the expression of such genes was therefore inconclusive, but is unlikely to be of biological significance.

6 Summary and conclusions

6.1 Overview

An overview of the whole study, linking the context with the aims, hypotheses and results and conclusions, is shown as a schematic in Figure 6.1, p 232.

6.2 Context

In recent decades, the ready availability of palatable, energy-rich foods and a reduction in physical activity have contributed to a huge global rise in the incidence of obesity and overweight [WHO, 2015]. These are associated with a wide range of diseases and disorders [Kopelman, 2007], and are characterised by excessive or abnormal fat accumulation [WHO, 2015]. Adipose tissue, the physical manifestation of obesity and overweight, is found in two main forms: white (WAT), which stores energy; and brown (BAT), which dissipates energy as heat by means of a unique protein, UCP1. A subset of adipocytes in WAT also express UCP1, and are termed brite/beige. In large mammals, BAT is rapidly replaced by WAT after birth [Cannon & Nedergaard, 2004]. However, it has recently been found that functional BAT is present in human adults [Cypess *et al*, 2009; van Marken Lichtenbelt *et al*, 2009; Virtanen *et al*, 2009], which raises the possibility that it could be manipulated to burn off excess fat. It is well established that the early life environment, particularly nutrition, can affect an adult's risk of obesity and its associated diseases [Fall, 2011; Martin-Gronert & Ozanne, 2013; Spencer, 2012], and it is possible that such 'programming' is mediated, in part at least, by the effects of early life nutrition on the deposition and development of WAT and/or BAT [Symonds *et al*, 2012b].

6.3 Aims

The principal aim of this study was to investigate, using sheep as a model, the effect of early nutritional interventions on growth of fat mass and the expression in adipose tissue

of genes involved in adipogenesis, energy metabolism, thermogenesis and development. A secondary aim was to study their ontogeny in sternal adipose tissue. A final aim was to determine whether UCP1-expressing adipocytes in the sternal adipose tissue of sheep were brown or brite/beige.

Context

Adipose tissue is found in two main forms: white (WAT) which stores energy; and brown (BAT), which dissipates energy as heat. The main site of BAT in adult humans is the supraclavicular depot. An equivalent site, the sternal depot, has recently been identified in sheep.

Human and animal studies indicate that fetal under- and overnutrition, and early postnatal overnutrition, programme for adult obesity. Timing of the nutritional challenge is a factor, but past results are inconsistent, and effects may be species- and/or sex-specific. Nutritional programming may be mediated, at least in part, by changes in adipose tissue biology.

Aims

To study the ontogeny of gene expression in the sternal adipose tissue of sheep over the first month of postnatal life.

To investigate, using sheep as a model, the effect of early nutritional interventions on growth of fat mass and gene expression in (primarily sternal) adipose tissue.

Study A, Chapter 3 **Study A, Chapter 4** **Study B, Chapter 5**

Hypotheses

The ontogeny of sternal adipose tissue would be similar to that of perirenal adipose tissue. Expression of adipogenic and metabolic genes would increase (↑), and that of thermogenic genes decrease (↓).

Fat supplements fed to lactating ewes would increase (↑) fat mass and the expression of adipogenic, metabolic and thermogenic genes in the sternal adipose tissue of suckling lambs.

Late gestational under- and overnutrition would change the expression (↑ or ↓) of adipogenic, metabolic, thermogenic and developmental genes in the sternal and subcutaneous adipose tissue of 6 m-old lambs.

A postnatal HCHF diet would increase (↑) the expression of adipogenic, metabolic and thermogenic genes in the sternal and subcutaneous adipose tissue of 6 m-old lambs.

Results and conclusions

Ontogeny of sternal adipose tissue was similar to that of perirenal. Metabolic genes ↑ , thermogenic genes ↓ , adipogenic genes varied (↑ , ↕ , ↓).

Sunflower oil: relative fat mass and leptin plasma levels ↑ in 28 day-old females. Canola oil: leptin gene expression and plasma levels ↑ in 28 day-old females.

Modifications to late gestational nutrition had no effect on fat mass or gene expression (↔).

Adipogenic genes ↓ , metabolic genes ↓ , thermogenic genes ↔ . Effect may be due to dedifferentiation of adipocytes following inflammation.

Figure 6.1 Overview of the study.

6.4 Methods

The work was carried out in two separate studies. The first study (Study A) examined the effect of fat supplements given to lactating ewes on gene expression in the sternal adipose tissue of their suckling lambs. Ewes were allocated to one of three feeding groups, one control and two supplemented, for 28 days after parturition, and their lambs were sampled at 7 and 28 days of age. Gene expression was determined by qPCR, and expression of UCP1 protein at 7 days of age was also assessed by western blotting, histology and immunohisto-chemistry. A number of 1 day-old lambs were also sampled and these, together with 7 and 28 day-old lambs from the control group, were used to examine the ontogeny.

The second study (Study B) looked at the effect of late gestational and early postnatal nutrition on gene expression in the sternal and subcutaneous adipose tissue of six month-old lambs. Twin-pregnant ewes were divided into three nutritional groups for the last six weeks of gestation: undernourished, control or overnourished. After parturition, one lamb from each twin pair was fed a control (roughage) diet, and the other a high-carbohydrate, high-fat (HCHF) diet. A subset of the lambs was sampled at six months of age, and gene expression was determined by qPCR.

6.5 Findings

6.5.1 Ontogeny of ovine sternal adipose tissue

It was found that the ontogeny of gene expression in sternal adipose tissue over the first month after birth was similar to that which has been reported for perirenal adipose tissue [Basse *et al*, 2015; Pope *et al*, 2014], with the main exception being the lipid droplet protein-encoding gene CIDEA, expression of which was unchanged in sternal adipose tissue, but reportedly declined with age in perirenal adipose tissue. CIDEA is a marker of BAT in mice, but is highly expressed in WAT in humans [Nordström *et al*, 2005; Zhou *et al*, 2003], so its expression pattern seems to be both tissue- and species-specific. Expression of most other thermogenic genes declined with age in sternal adipose tissue, as expected. The expression of most metabolic genes increased with age, again as expected, while that of adipogenic genes was more varied. Notably, there was a disparity in the expression profiles of the two principal regulators of adipogenesis, PPARγ and C/EBPα, with expression of the former increasing with age, and that of the latter peaking at 7 days of age, before declining again to a 1-day level. As these two genes promote each other's expression [Rosen *et al*, 2002], the finding was somewhat surprising. The expression of another important adipogenic gene, SREBF1, which would be expected to correlate with that of PPARγ and C/EBPα, declined

with age. The two studies cited above did not test expression of either C/EBPα or SREBF1 in perirenal adipose tissue, so it is unclear whether these unexpected expression patterns are general or tissue-specific.

6.5.2 Effect of late gestational undernutrition on birth weight of lambs

It was found that male, but not female, offspring of mothers undernourished during the last six weeks of gestation had lower birth weights than the offspring of normally fed or overnourished mothers, but that the body weight of these animals had 'caught up' to that of their peers by six months of age. While there is good support for a relationship between late gestational undernutrition and reduced birthweight, at least in sheep [Kenyon & Blair, 2014], little information could be found about sex-specific effects, particularly in humans. The only study in sheep reporting such an effect found that the female offspring of ewes undernourished in late gestation had a higher birth weight than males, but there were substantial differences in the experimental protocol [Oliver *et al*, 2002].

6.5.3 Effects of maternal fat supplements on the sternal adipose tissue of suckling lambs

A sunflower, but not a canola, oil supplement fed to lactating ewes increased the adipose tissue weight, relative to body weight, of female, but not male, suckling lambs at 28 days of age. This could be a function of their respective mix of fatty acids. Sunflower oil contains more pro-adipogenic [Ailhaud *et al*, 2006; Muhlhausler & Ailhaud, 2013] ω-6 fatty acids than canola oil, while canola oil, unlike sunflower oil, contains a substantial proportion of anti-obesogenic [Buckley & Howe, 2010; Flachs *et al*, 2009] ω-3 fatty acids [FAO, 1999]. A potential mechanism for the lack of effect in males is a less efficient conversion of the ω-6 linoleic acid to the more adipogenic ω-6 arachidonic acid. The study was considerably underpowered, however, so it is conceivable that canola oil has a similar, if lesser effect, to that of sunflower oil in females. There was some indication that fat supplements increased the expression of adipogenic and metabolic genes in the sternal adipose tissue of female lambs, but the evidence was generally relatively weak and inconclusive. However, there was good evidence that sunflower oil increased mRNA expression of the adipokine leptin in female lambs at 28 days of age, with weaker evidence of a similar effect with canola oil. Both supplements also increased the plasma concentration of leptin at 7 and 28 days of age in female, but not male, lambs. The similar pattern of sexual dimorphism found in relative adipose tissue weight, leptin mRNA expression and leptin plasma concentration at 28 days of age concurs with previous reports that leptin plasma concentration and gene expression

generally correlate to WAT mass [Ahima & Flier, 2000; Friedman & Halaas, 1998]. Finally, a canola oil supplement increased expression of a number of genes, including UCP1, in 7 day-old male lambs relative to females. However, the effect was not well supported by UCP1 protein expression analysis, and had disappeared by 28 days of age.

6.5.4 Effects of an obesogenic diet on 6 month-old lambs

The obesogenic HCHF diet increased adipose tissue weight in all depots measured at six months of age, with the greatest effect in the perirenal depot. Female lambs had more perirenal adipose tissue relative to body weight than males, which is consistent with previous reports of a similar effect in younger lambs [Duffield *et al*, 2009; Wallace *et al*, 2014]. Contrary to what had been expected, an HCHF diet substantially reduced the expression of most adipogenic and metabolic genes in sternal and subcutaneous adipose tissue, a phenomenon which has been previously reported for some murine adipose tissue depots [Moraes *et al*, 2003; Nadler *et al*, 2000], and which may be the result of the dedifferentiation of adipocytes arising from the increased expression of inflammatory genes, notably TNFα [Al-Hasani & Joost, 2005; Gustafson *et al*, 2009; Sethi & Hotamisligil, 1999]. In support of this hypothesis, colleagues found that the HCHF diet increased the expression of a number of inflammatory genes, including TNFα, in the adipose tissues that they tested (which did not include sternal). The HCHF diet had different effects on the expression of leptin and RIP140 in sternal and subcutaneous adipose tissue. In the former tissue, there was no change in expression of either gene, but in the latter, the HCHF diet upregulated leptin and down-regulated RIP140. Expression of thermogenic genes was barely detectable in either tissue.

6.5.5 Cross-study observations

The most notable difference between the two nutritional studies was that there was very little evidence of sexual dimorphism in Study B (Chapter 5). The fat supplements fed to lactating ewes in Study A (Chapter 4) generally affected their female, but not male, suckling lambs. In contrast, the obesogenic diet fed to lambs in Study B for 6 months seemed to have a similar effect on males and females. The difference between the studies may be a function of different processes at work, with the effects seen in Study A being the result of differentiation, and those seen in Study B being the result of dedifferentiation.

The main similarity between the two studies was in the overall relative expression of the genes tested, ignoring nutritional interventions, across tissues and time points.[40] In all

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[40] It should be noted that relative expression at 6 months of age is only an estimate based on the C_T value from qPCR, as absolute quantification was not used in Study B.

three tissues examined (sternal adipose tissue in Studies A and B and subcutaneous adipose tissue in Study B), and at all three time points (7 days, 28 days and 6 months), FABP4 was by far the most highly expressed gene, followed by adiponectin and PPARγ, then usually by some combination of C/EBPα and NR3C1. That pattern was only broken by UCP1, which was the third most highly expressed gene at 7 days of age, and ATF2, which was the fourth most highly expressed gene in subcutaneous adipose tissue at 6 months of age. Most other thermogenic genes were expressed at very low levels, even at 7 days of age.

6.5.6 Summary of findings

In conclusion, the ontogeny of gene expression in the sternal adipose of newborn lambs in the first month after birth was similar to that reported for perirenal adipose tissue. The expression of most thermogenic genes declined with age, as expected, but the expression profiles of several important adipogenic genes were surprisingly varied. Late gestational undernutrition reduced the birth weight of male lambs, but their body weight had caught up to that of their peers by six months of age. Fat supplements fed to ewes during lactation increased adipose tissue weight, plasma leptin concentration and leptin mRNA expression (in sternal adipose tissue) at 28 days of age, but only in female lambs. There was evidence of a similar effect for adipogenic and other metabolic genes, and sunflower oil seemed to have a greater effect than canola oil in some cases. In contrast, an HCHF diet fed to new-born lambs over a 6-month period considerably reduced the expression of adipogenic and metabolic genes in the sternal and subcutaneous adipose tissue of both males and females, notwithstanding substantial increases in adipose tissue weight.

6.6 Limitations

6.6.1 Use of sheep as a model for human development

The advantages of using a sheep model were set out in Section 1.5, p 50. However, while the sheep is a well-established model in neonatology, it has obvious disadvantages for a nutritional study in that it is a herbivore and ruminant, so does not metabolise energy in the same way as a monogastric species such as a human. The problem is largely mitigated in Study A in that the rumens of lambs are not fully developed until some 6–8 weeks after birth, metabolic development of the rumen is in any event dependent on solid feed [Lane et al, 2000], and lambs were milk-fed by their mothers for the duration of the study (being a maximum of 28 days). Additionally, suckling elicits the oesophageal groove reflex [Comline & Titchen, 1951; Ørskov & Benzie, 1969], enabling most of the milk to bypass the rumen

and flow directly into the stomach. The problem was partially mitigated for the HCHF diet in Study B, as described in Section 5.3.1.4, p 198, by using a suckling bucket fitted with a rubber teat to deliver the milk-cream mixture, thereby triggering the oesophageal groove reflex, and by feeding maize, which degrades more slowly in the rumen than other cereals [Ørskov, 1986; Ørskov *et al*, 1969].

6.6.2 Technical limitations of a sheep model

Sequencing of the sheep genome has only recently been completed [Jiang *et al*, 2014], and in the early stages of the study primers had to be designed to the cow genome. Attempts to design primers for some genes of interest (eg the important adipogenic gene C/EBPβ and the classical BAT marker ZIC1) were unsuccessful. Furthermore, only a limited number of commerical antibodies are available for sheep, so protein expression testing was restricted to UCP1.

6.6.3 Fetal number

It is known that fetal number has an effect on birth weight [Gardner *et al*, 2007], and it has been reported that twins have a higher percentage fat mass at 2 years of age [Hancock *et al*, 2012]. It has also been found that triplets have a higher 'dressing yield' at around 6 months of age than twins and singletons [Afolayan *et al*, 2007]. Fetal number therefore clearly has an effect on postnatal body composition, so the inclusion of 10 triplet-born lambs with the 44 twin-born lambs in Study A is a potential confounding factor.

6.6.4 Rearing number

In Study A, one lamb from each twin-pair, or pair of surviving triplets, was sampled at around 7 days of age, and the other at around 28 days of age. Lambs were therefore raised in pairs from 1–7 days of age and alone from 8–28 days of age. The growth rate, blood pressure control and glucose homeostasis of a twin-born lamb raised alone by its mother is equivalent to that of a singleton [Symonds & Budge, 2009]. Lambs in each sampling age group were therefore exposed to different nutritional environments, which is a potential confounding factor in the ontogeny study.

6.6.5 Fat supplements

The fat supplements used in Study A were supplements in the true sense of the word, in that they were additions to a diet rather than replacements. While this is reasonable from a translational perspective (ie it is much easier to get patients to take supplements than

replace items in their diet), it means that the observed effects could be attributable simply to more fat in the diet rather than the unique characteristics of sunflower or canola oil.

6.6.6 Sex balance

There was a sex imbalance in Study B in that there were only males in the prenatal control group ('normal nutrition', N) for lambs sampled at six months of age. This was a function of the fact that there fewer males than females overall in the prenatal control group, and that priority was given to achieving a sex balance at a later sampling date (not reported in this study). This informed the decision to report the results of Study B on a consolidated basis rather than by individual sex.

6.6.7 Histology and immunohistochemistry

In Study A, there were problems with some of the histological sections (eg connective tissue only, too many layers of cells) and with the immunohistochemistry (eg cover slips coming off some slides during processing). Unfortunately there was insufficient time to repeat the defective or missing samples.

6.6.8 Statistical power

While statistical power was around the conventionally accepted level of 80 % for (most of) the ontogeny part of Study A, and Study B was only slightly underpowered (around 66 %), the nutritional part of Study A was considerably underpowered in almost all cases, with power only exceeding 50 % in one instance, and often being in single percentage figures. It was calculated that sample sizes of 20 would be required for each age/feeding group/sex combination to achieve a power of 80 % with a reasonable effect size of 1.0, which would require a total of 240 animals. This is clearly not realistic for a large mammal study.

6.7 Alternative techniques

6.7.1 RNA integrity

Analysis of RNA integrity by simple gel electrophoresis (Section 2.4.5, p 92) is very crude, and its interpretation is subjective. There are more sophisticated methods available that give a quantitative indication of integrity, though inevitably they come at a cost. The most widely-used method is the Agilent Bioanalyzer, a 'chip'-based system that uses electrophoresis to separate RNA fragments via interconnected microchannels fabricated in glass.

Samples are allocated an RNA Integrity Number (RIN), ranging from 1 (totally degraded) to 10 (intact) [Schroeder *et al*, 2006].

6.7.2 Gene selection and expression

Instead of selecting a list of candidate genes subjectively and analysing their expression by qPCR, a more targeted approach would have been to use genome-wide techniques to identify the genes with the greatest fold changes in expression under different nutritional conditions. These could then have been subjected to confirmatory testing using qPCR. The traditional method for measuring the expression of multiple genes simultaneously is the DNA microarray, a hybridisation-based system requiring an array or 'chip' of species- and transcript-specific probes, but it has limited chip availability in sheep. It has also now been largely superseded by 'RNA sequencing' (RNA-Seq), which uses next generation sequencing (NGS) technologies, and which has a number of advantages over microarrays, including no requirement for a predetermined transcriptome, and increased sensitivity and specificity [Wang *et al*, 2009]. However, both DNA microarrays [Thompson *et al*, 2007] and RNA-Seq [Van Verk *et al*, 2013] are relatively expensive, and both usually require a higher level of RNA integrity than qPCR (RIN ≥ 8), which might have been a problem in this study.

6.7.3 Protein expression

Enzyme-linked immunosorbent assay (ELISA) could have been used as an alternative to western blotting for the analysis of protein expression, but as the protein is not denatured using this technique, epitope sites can be concealed, and it can therefore be more difficult to find suitable antibodies. If no antibodies are readily available for a protein of interest, a further alternative technique would have been quantitative mass spectrometry. Although this technique is usually based on the incorporation of stable isotopes or 'mass tags' into samples, limiting the number of samples that can be compared, unlabelled variants such as spectral counting, peak measurements quantification, and selected reaction monitoring (SRM) can be used to compare multiple samples [Rodríguez-Suárez & Whetton, 2013].

6.7.4 Histology

With more available time, the number and mean size of adipocytes from each sample could potentially have been calculated using stereology, which is the 3-dimensional analysis of a series of histological tissue sections that are cut at defined, regular intervals. It is more accurate than 2-dimensional imaging/counting techniques, and reduces selection bias. A potential problem, however, is that the lipid content of adipocytes makes it difficult to

reliably slice high-quality tissue sections from the wax blocks (ie they tend to crumble), so obtaining consecutive sections of sufficient quality might have been impractical.

6.8 Future work

6.8.1 Ontogeny of sternal adipose tissue

Work on the ontogeny of sternal adipose tissue was limited to looking at fat mass and gene expression. Ideally, this needs to be supported by work on protein expression, particularly in relation to the diverse expression patterns of the adipogenic genes PPARγ, C/EBPα and SREBF1. However, as mentioned in Section 6.6.2, p 237, this is dependent on the availability of suitable antibodies. It would also be informative to look at the gene expression profiles of other important adipogenic genes, such as C/EBPβ and C/EBPδ, and of the lipid droplet protein-encoding gene CIDEC/FSP27, which is closely related to CIDEA. CIDEA itself showed some suggestion of sexual dimorphism in its expression pattern, and ideally further work would be undertaken to confirm or refute this.

6.8.2 Extension of ontogeny study to other tissues and species

The most well-defined role of CIDEA in adipose tissue is the enlargement of lipid droplets, in conjunction with CIDEC/FSP27, by mediating the transfer of triglycerides from smaller to larger droplets. Its level of expression in BAT and WAT is species-specific, but has not been fully characterised in sheep. It would be useful, therefore, to examine the ontogeny of CIDEA and CIDEC/FSP27 in other ovine adipose tissues. It would also be informative, in view of their diverse and unexpected expression patterns in sternal adipose tissue, to look at the early ontogeny of the key adipogenic regulators PPARγ, C/EBPα and SREBF1 in other ovine adipose tissues, and potentially other species, to ascertain whether such patterns are tissue- and/or species-specific.

6.8.3 Effect of late gestational undernutrition on birth weight of lambs

Male, but not female, offspring of ewes undernourished during late gestation had lower birthweights than offspring of 'normally' fed or overnourished ewes. Future studies will be required to ascertain whether this apparent maintenance of 'normal' birth weight in the female offspring of undernourished mothers is replicated,[41] and if so, to find out whether it correlates with an improved prognosis in adulthood, to what extent the timing and

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[41] One previous study reports an opposite effect, with the female, but not male, progeny of undernourished mothers having lower birth weights than controls [Oliver *et al*, 2002].

degree of nutritional restriction is a factor, and what the possible mechanisms might be for the protective effect in females.

6.8.4 Effect of maternal fat supplements on suckling lambs

Study A found evidence of sexual dimorphism in the effects of maternal fat supplements on the adipose tissue weight, plasma leptin concentration, rectal temperature and gene expression of suckling lambs. There was also an indication that in some cases sunflower oil had more effect than canola oil. However, the study was considerably underpowered, and ideally it would be repeated with larger sample sizes to confirm the observed effects. In any event, further work needs to be carried out to support or explain the findings. The protein expression and histology work done on UCP1 at 7 days of age needs to be extended to 28 days of age, particularly as a colleague has found (currently unpublished) evidence that canola oil increased the expression of UCP1 protein in perirenal adipose tissue at that age, notwithstanding the fact that there was no change in mRNA expression [Woods *et al*, 2015]. Ideally the protein analysis work would be extended to other genes, particularly leptin, but this would be subject to the availability of the necessary antibodies. Finally, it was postulated that the apparent differential effects of sunflower oil and canola oil might be attributable to their respective mix of ω-3 and ω-6 fatty acids. Analysis of the fatty acid content of the ewes' milk (which was sampled) would provide evidence to support or refute this hypothesis.

6.8.5 Effect of an obesogenic diet on 6 month-old lambs

It has been postulated that the substantial reduction in expression of adipogenic genes in the sternal and subcutaneous adipose tissue of 6 month-old lambs might be a function of the dedifferentiation of adipocytes caused by macrophage infiltration and an increase in the expression of TNFα and other inflammatory genes. Expression of inflammatory genes has not been tested in sternal adipose tissue, however, so future work will be required to rectify that omission, particularly in respect of TNFα. It might also be informative to test the expression of genes from the NFκB family, which regulate numerous genes involved in inflammation [Hoesel & Schmid, 2013; Lawrence, 2009]. An obesogenic diet had a different effect on the expression of leptin, RIP140, ATF2 and HOXC9 in the two tissues, and its effect on leptin in subcutaneous adipose tissue had some suggestion of sexual dimorphism, so it would be useful to test expression of these genes in other adipose tissues (ie perirenal, epicardial and mesenteric) to get a more comprehensive view of tissue- and possibly sex-specific differences in their expression.

6.9 Final remarks

This study has examined the effect of nutritional interventions at various stages of early life on adipose tissue in sheep, and has found that such interventions affect adipose tissue weight and the expression of adipogenic, metabolic and some developmental genes. Some early interventions showed evidence of sexual dimorphism, while a more sustained post-natal intervention did not. Future studies will be required to explain these phenomena, to ascertain what effect they have on the adult phenotype and health outcomes, and to assess their implications for humans.

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B Chapter 3: supplementary information

B.1 Tissue weights by sex and age

Sternal and perirenal adipose tissue weights by sex and age are shown in Figure B-1, p 283.

B.2 Gene expression by sex and age

Gene expression in sternal adipose tissue by sex and age is shown in Figure B-2, p 284 (adipogenic genes), Figure B-3, p 285 (metabolic genes), Figure B-4, p 286 (thermogenic genes) and Figure B-5, p 287 (developmental genes).
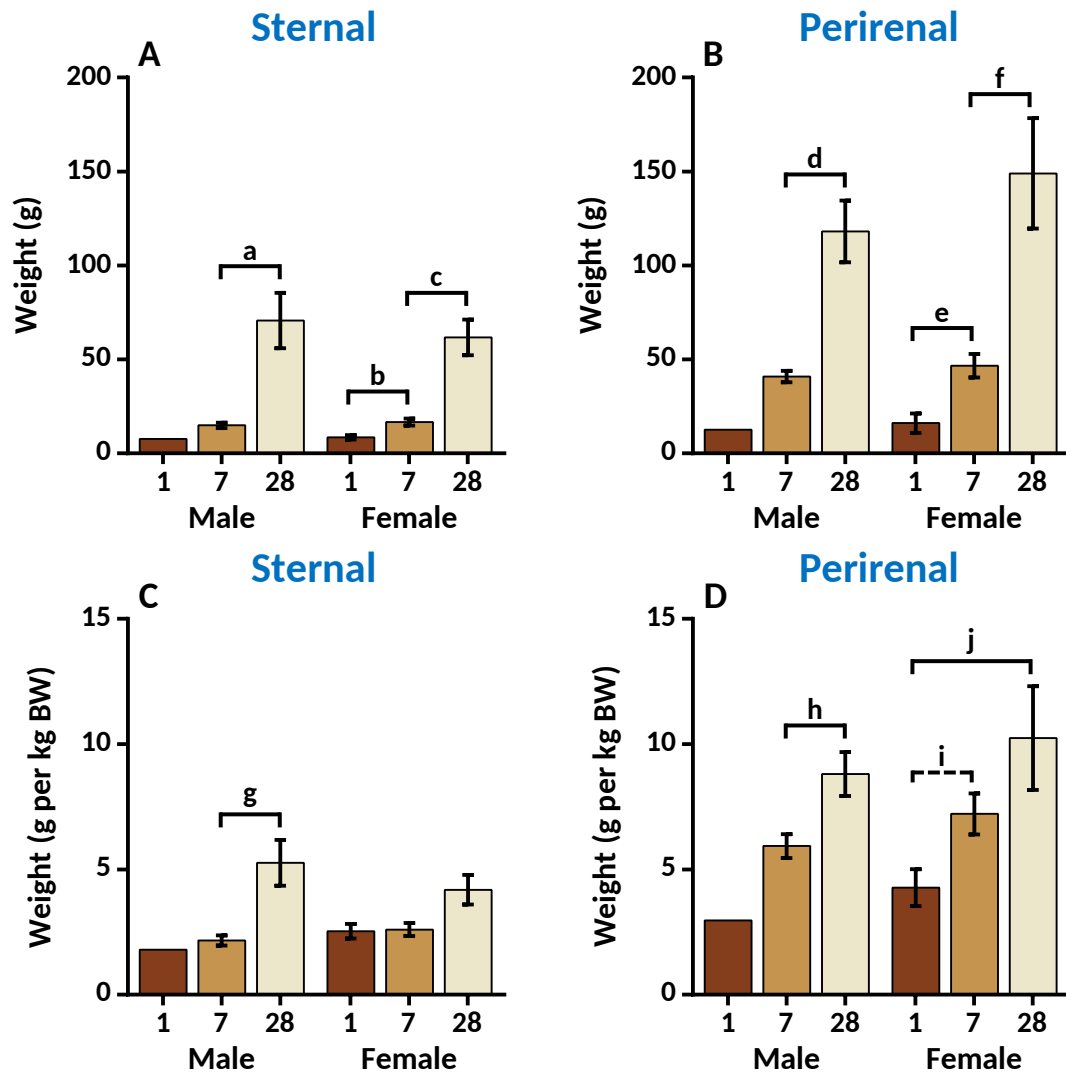
Figure B-1 Sternal and perirenal adipose tissue weights by sex and age. Values shown are mean tissue weights ± SEM of lambs sampled at 1 (+ 2; M: n = 1; F: n = 3/4), 7 (± 1; M/F: n = 4) and 28 (± 3; M: n = 4; F: n = 5) days of age, both in absolute terms (**A**, **B**) and relative to body weight (BW) (**C**, **D**). M is male, F is female. The 1–7 and 7–28 groups were compared by Mann-Whitney U-tests, with the 1–28 groups also being compared if p > 0.05 for both the first two tests (a: p = 0.0286; b: p = 0.0286; c: p = 0.0159; d: p = 0.0286; e: p = 0.0286; f: p = 0.0159; g: p = 0.0286; h: p = 0.0286; i: p = 0.0571; j: p = 0.0357; p > 0.1 in all other cases).
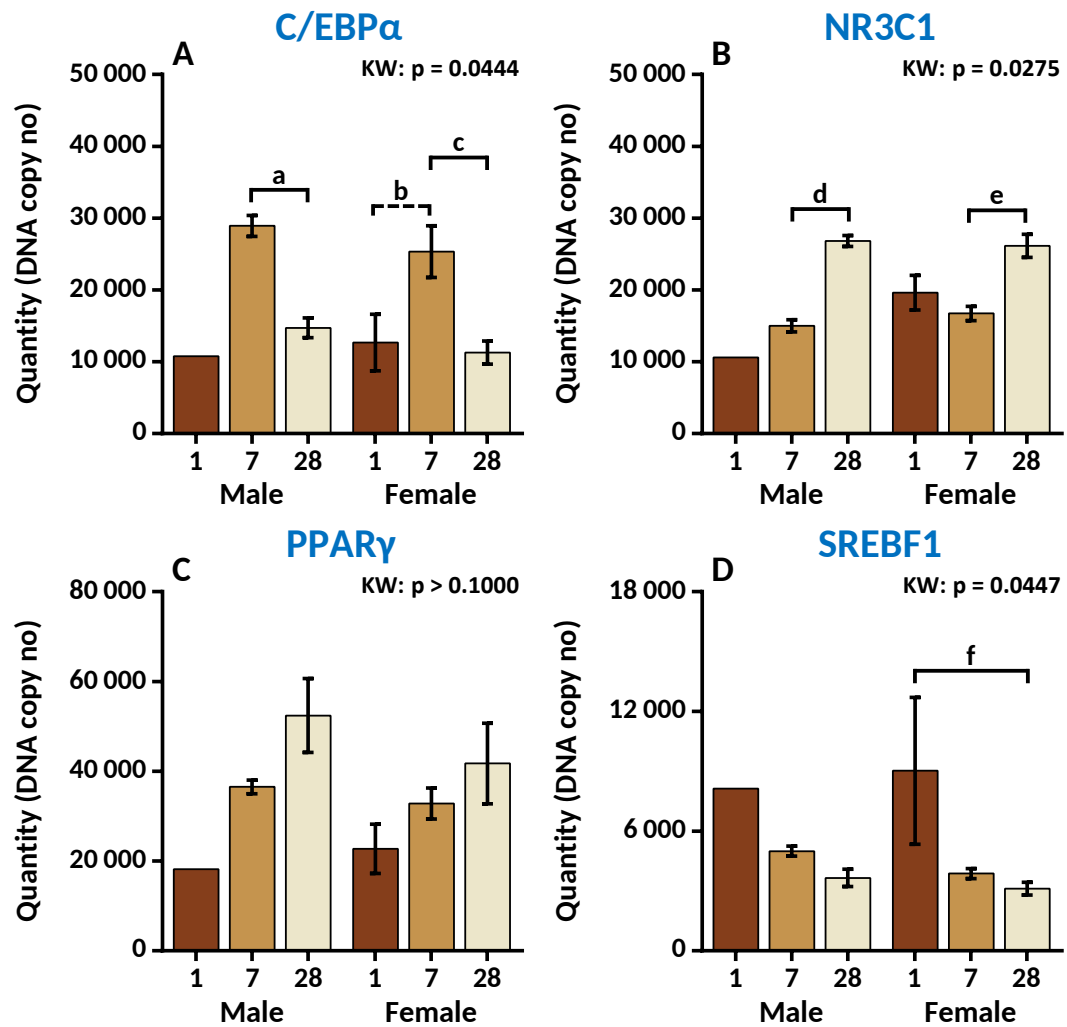
Figure B-2 Expression of adipogenic genes in sternal adipose tissue by sex and age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; M: n = 1; F: n = 4), 7 (± 1; M/F: n = 4) and 28 (± 3; M: n = 4; F: n = 5) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of IPO8, KDM2B, RPLP0 and TBP. For males, the 7- and 28-day groups were compared by Mann-Whitney U-test (a: p = 0.0286; d: p = 0.0286). For females, age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (b: p = 0.0566; c: p = 0.0176; e: p = 0.0083; f: p = 0.0128). No adjustments have been made to p-values for multiple comparisons.
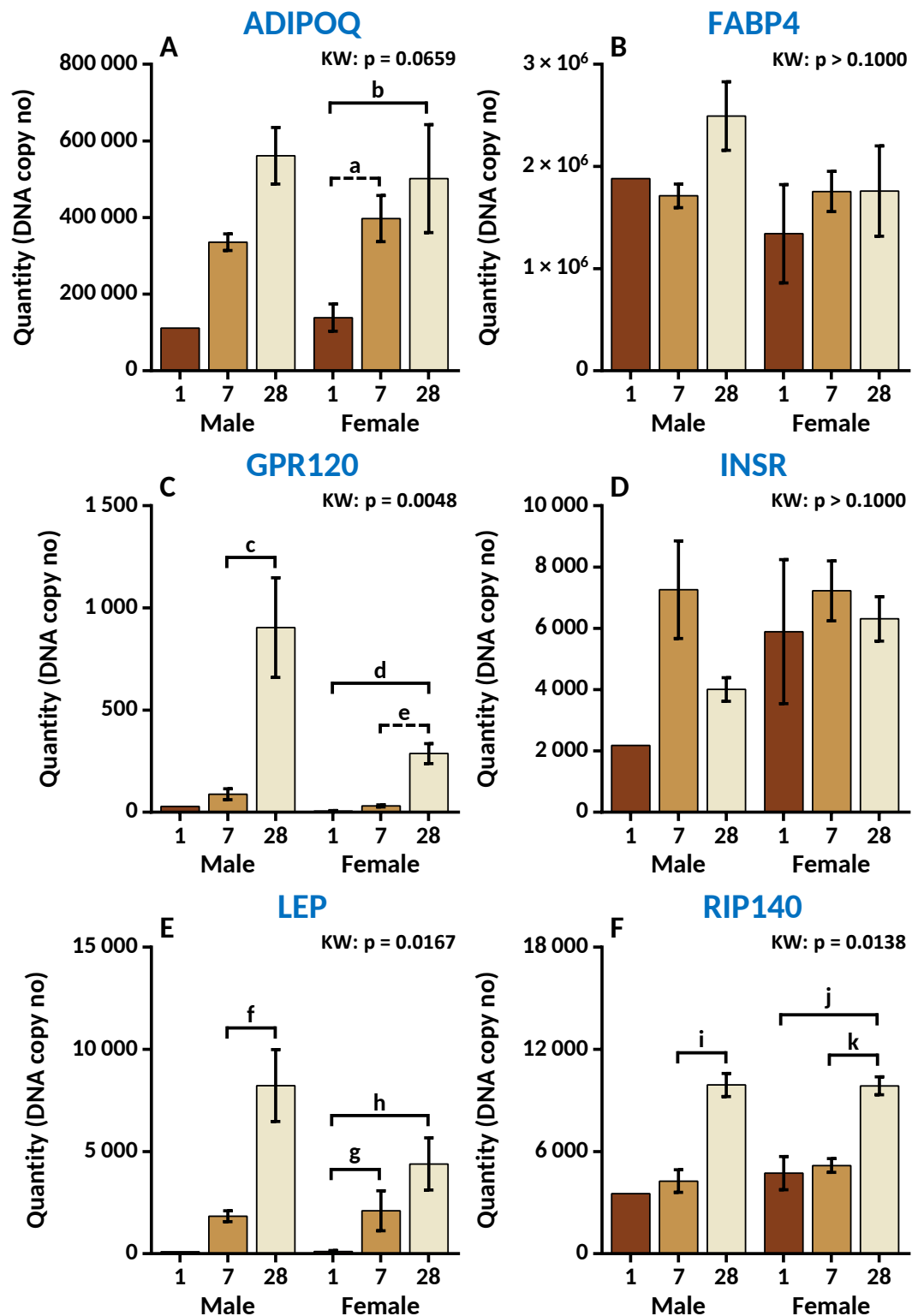
Figure B-3 Expression of metabolic genes in sternal adipose tissue by sex and age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; M: n = 1; F: n = 4), 7 (± 1; M/F: n = 4) and 28 (± 3; M: n = 4; F: n = 5) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of IPO8, KDM2B, RPLP0 and TBP. For males, the 7- and 28-day groups were compared by Mann-Whitney U-test (c: $p = 0.0286$; f: $p = 0.0286$; i: $p = 0.0286$). For females, age groups were compared by Kruskal-Wallis (KW) test and, if $p < 0.1$, by *post hoc* Dunn's tests (a: $p = 0.0694$; b: $p = 0.0277$; d: $p = 0.0011$; e: $p = 0.0850$; g: $p = 0.0458$; h: $p = 0.0052$; j: $p = 0.0128$; k: $p = 0.0128$). No adjustments have been made to p-values for multiple comparisons.
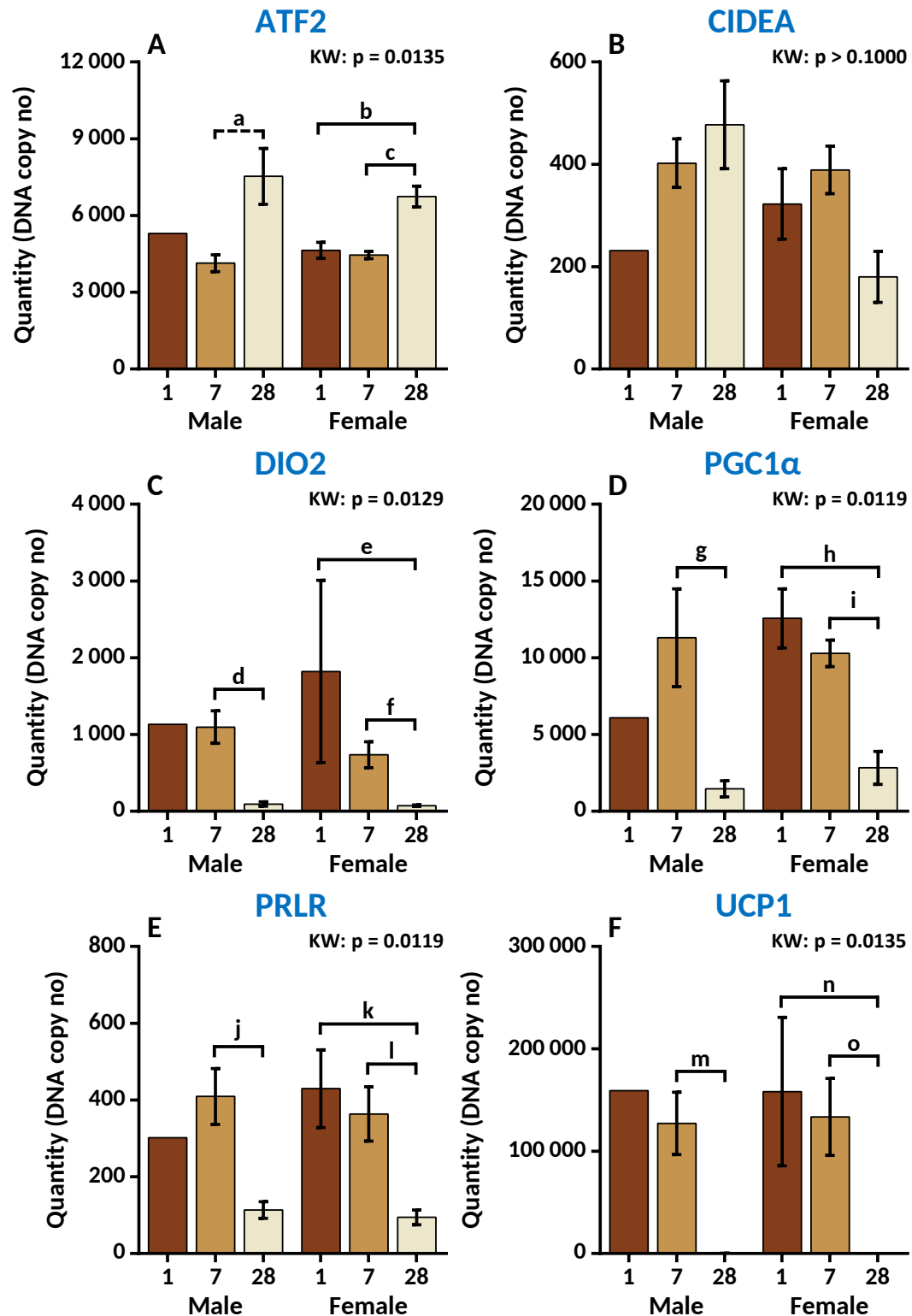
Figure B-4 Expression of thermogenic genes in sternal adipose tissue by sex and age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; M: n = 1; F: n = 4), 7 (± 1; M/F: n = 4) and 28 (± 3; M: n = 4; F: n = 5) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of IPO8, KDM2B, RPLP0 and TBP. For males, the 7- and 28-day groups were compared by Mann-Whitney U-test (a: p = 0.0571; d: p = 0.0286; g: p = 0.0286; j: p = 0.0286; m: p = 0.0286). For females, age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (b: p = 0.0167; c: p = 0.0098; e: p = 0.0074; f: p = 0.0216; h: p = 0.0055; i: p = 0.0277; k: p = 0.0055; l: p = 0.0277; n: p = 0.0098; o: p = 0.0167). No adjustments have been made to p-values for multiple comparisons.
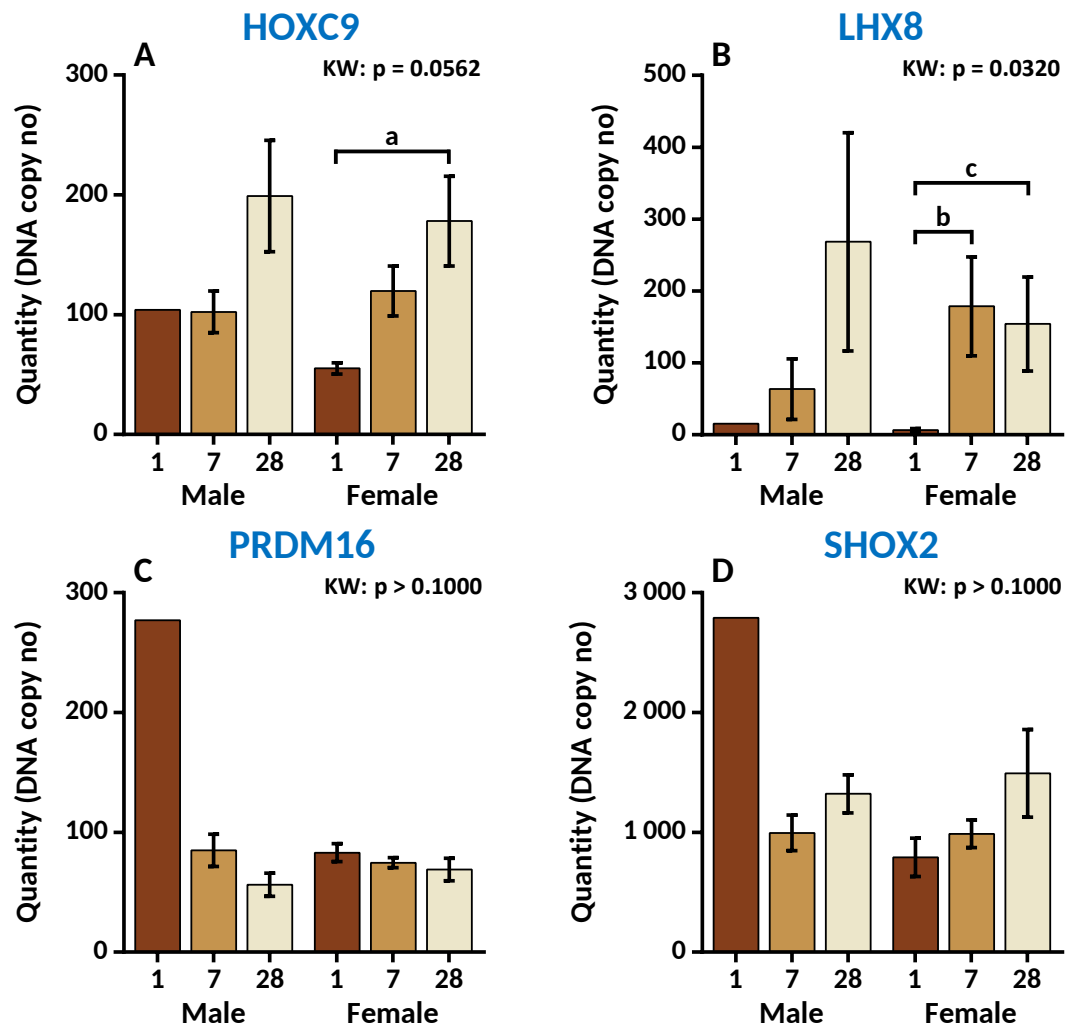
Figure B-5 Expression of developmental genes in sternal adipose tissue by sex and age. Values shown are mean normalised DNA copy number ± SEM of genes from lambs sampled at 1 (+ 2; M: n = 1; F: n = 4), 7 (± 1; M/F: n = 4) and 28 (± 3; M: n = 4; F: n = 5) days of age; M is male, F is female. Expression of genes of interest was normalised to the geometric mean expression of IPO8, KDM2B, RPLP0 and TBP. For males, the 7- and 28-day groups were compared by Mann-Whitney U-test (p > 0.1 in all cases). For females, age groups were compared by Kruskal-Wallis (KW) test and, if p < 0.1, by *post hoc* Dunn's tests (a: p = 0.0186; b: p = 0.0232; c: p = 0.0206). No adjustments have been made to p-values for multiple comparisons.

C Chapter 4: supplementary information

C.1 Body weights by sex and feeding group

Mean body weights at birth and at 7 days of age by sex and feeding group for all lambs (including those sampled at 28 days of age) are shown in Table C-1, below.

Table C-1 Body weights. Values shown are mean body weights ± SEM by feeding group and sex for all lambs (including those sampled at 28 days). Feeding groups are: control (N); sunflower oil supplement (S); canola oil supplement (C). M is male, F is female. For males, n = 7–8 for all groups; for females, n = 8–10 for all groups. S and C groups were compared to the appropriate N group by Mann-Whitney U-tests (p > 0.1 in all cases).

Age	Sex	Weight (kg)		
		N	S	C
Birth	M	4.64 ± 0.17	5.20 ± 0.32	4.80 ± 0.21
	F	5.05 ± 0.35	4.67 ± 0.21	5.39 ± 0.21
7 days	M	6.62 ± 0.24	7.18 ± 0.32	7.12 ± 0.22
	F	6.84 ± 0.33	7.10 ± 0.36	7.25 ± 0.24

D Chapter 5: supplementary information

D.1 Body and tissue weights

Birth weights and body weights at six months by postnatal feeding group and sex are shown in Table D-1, below. Adipose tissue weights at six months by prenatal feeding group and sex are shown in Table D-2, p 290 (absolute) and Table D-3, p 290 (relative). Adipose tissue weights at six months by postnatal feeding group and sex are shown in Table D-4, p 291 (absolute) and Table D-5, p 291 (relative).

D.2 Gene expression

Gene expression by prenatal group and sex is shown in Table D-6, p 292 (sternal adipose tissue, male), Table D-7, p 293 (sternal adipose tissue, female), Table D-8, p 294 (subcutaneous adipose tissue, male) and Table D-9, p 295 (subcutaneous adipose tissue, female). Gene expression by postnatal group and sex is shown in Table D-10, p 296 (sternal adipose tissue, male), Table D-11, p 297 (sternal adipose tissue, female), Table D-12, p 298 (subcutaneous adipose tissue, male) and Table D-13, p 299 (subcutaneous adipose tissue, female). The effect of outlier removal on gene expression by postnatal group and sex is shown in Table D-14, p 300 (male) and Table D-15, p 300 (female).

Table D-1 Birth weights of lambs and body weights at 6 months by postnatal feeding group and sex. Values shown are mean body weights ± SEM. Feeding groups are: control (C: n = 35; 16M, 19F); high-carbohydrate, high-fat (HCHF) (H: n = 35; 18M, 17F); M is male, F is female. Postnatal feeding groups within each sex were compared by Mann-Whitney U-tests (a: p = 0.0600; p > 0.1 in all other cases).

Item	Sex	Weight (kg)	
		C	H
Birth weight	M	4.21 ± 0.19	4.30 ± 0.19
	F	4.16 ± 0.13	4.05 ± 0.20
Body weight at 6 months	M	38.03 ± 1.03 [a]	44.07 ± 2.08 [a]
	F	34.38 ± 0.66	37.74 ± 2.19

Table D-2 Absolute adipose tissue weights at 6 months by prenatal feeding group and sex. The values shown are mean tissue weights ± SEM. Feeding groups are: undernutrition (U: n = 10; 5M, 5F); 'normal' nutrition (N: n = 6; 6M, 0F); overnutrition (O: n = 10; 4M, 6F); M is male, F is female. For male lambs, all three groups were compared by Kruskal-Wallis test (p > 0.1 in all cases). For female lambs, the two groups were compared by Mann-Whitney U-test (p > 0.1 in all cases).

Tissue	Sex	Adipose tissue weight (g)		
		U	N	O
Mesenteric	M	811 ± 301	916 ± 406	757 ± 369
	F	686 ± 239		659 ± 203
Perirenal	M	1 096 ± 459	975 ± 400	899 ± 458
	F	1 062 ± 519		865 ± 308
Sternal	M	130 ± 41	116 ± 37	109 ± 48
	F	78.4 ± 26.3		81.6 ± 25.9
Subcutaneous	M	235 ± 92	259 ± 91	130 ± 60
	F	140 ± 51		136 ± 44
Combined	M	2 273 ± 882	2 265 ± 895	1 895 ± 928
	F	1 967 ± 826		1 741 ± 564

Table D-3 Relative adipose tissue weights at 6 months by prenatal feeding group and sex. The values shown are mean tissue weights ± SEM relative to body weight (BW). Feeding groups are: undernutrition (U: n = 10; 5M, 5F); 'normal' nutrition (N: n = 6; 6M, 0F); overnutrition (O: n = 10; 4M, 6F); M is male, F is female. For male lambs, all three groups were compared by Kruskal-Wallis test (p > 0.1 in all cases). For female lambs, the two groups were compared by Mann-Whitney U-test (p > 0.1 in all cases).

Tissue	Sex	Adipose tissue weight (g per kg BW)		
		U	N	O
Mesenteric	M	18.7 ± 5.8	17.9 ± 6.8	17.7 ± 8.4
	F	17.9 ± 6.3		19.3 ± 5.4
Perirenal	M	25.2 ± 9.3	19.4 ± 7.1	21.1 ± 10.8
	F	26.5 ± 12.5		26.7 ± 9.8
Sternal	M	3.13 ± 0.85	2.40 ± 0.64	2.53 ± 1.00
	F	2.03 ± 0.65		2.39 ± 0.67
Subcutaneous	M	5.42 ± 1.89	5.27 ± 1.59	3.01 ± 1.28
	F	3.60 ± 1.22		3.95 ± 1.12
Combined	M	52.5 ± 17.6	45.0 ± 15.3	44.3 ± 21.3
	F	50.2 ± 20.4		52.4 ± 16.4

Table D-4 Absolute adipose tissue weights at 6 months by postnatal feeding group and sex. Values shown are mean tissue weight ± SEM. Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF) (H: n = 13; 8M, 5F); M is male, F is female. The two groups within each sex were compared by Mann-Whitney U-test (p is shown in the right-hand column). Males and females within each tissue and feeding group were also compared by Mann-Whitney U-test (a: p = 0.0734; b: p = 0.0932; p > 0.1 in all other cases).

Tissue	Sex	Adipose tissue weight (g)		p
		C	H	
Mesenteric	M	182 ± 19 [a]	1 413 ± 223	0.0003
	F	303 ± 64 [a]	1 113 ± 154	0.0087
Perirenal	M	134 ± 11	1 749 ± 184	0.0003
	F	210 ± 43	1 848 ± 220	0.0043
Sternal	M	38.5 ± 2.8	189 ± 19 [b]	0.0003
	F	34.3 ± 4.4	135 ± 17 [b]	0.0043
Subcutaneous	M	47.6 ± 8.7	364 ± 47	0.0003
	F	56.5 ± 6.2	235 ± 34	0.0043
Combined	M	402 ± 38	3 715 ± 408	0.0003
	F	603 ± 114	3 332 ± 362	0.0043

Table D-5 Relative adipose tissue weights at 6 months by postnatal feeding group and sex. Values shown are mean tissue weight ± SEM relative to body weight (BW). Feeding groups are: control (C: n = 13; 7M, 6F); high-carbohydrate, high-fat (HCHF) (H: n = 13; 8M, 5F); M is male, F is female. The two groups within each sex were compared by Mann-Whitney U-test (p is shown in the right-hand column). Males and females within each tissue and feeding group were also compared by Mann-Whitney U-test (a: p = 0.0513; b: p = 0.0186; c: p = 0.0535; p > 0.1 in all other cases).

Tissue	Sex	Adipose tissue weight (g per kg BW)		p
		C	H	
Mesenteric	M	4.90 ± 0.50 [a]	29.7 ± 3.3	0.0003
	F	8.68 ± 1.80 [a]	30.7 ± 3.6	0.0043
Perirenal	M	3.60 ± 0.27	37.8 ± 2.7 [b]	0.0003
	F	5.98 ± 1.17	51.4 ± 4.0 [b]	0.0043
Sternal	M	1.04 ± 0.09	4.11 ± 0.30	0.0003
	F	0.99 ± 0.13	3.71 ± 0.28	0.0043
Subcutaneous	M	1.26 ± 0.20	7.74 ± 0.66	0.0003
	F	1.63 ± 0.19	6.38 ± 0.51	0.0043
Combined	M	10.8 ± 0.9 [c]	79.3 ± 4.8	0.0014
	F	17.3 ± 3.2 [c]	92.3 ± 6.2	0.0043

Table D-6 Gene expression of male lambs in sternal adipose tissue by prenatal feeding group. Feeding groups are: undernutrition (U: n = 5); 'normal' nutrition (N: n = 6); overnutrition (O: n = 4). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (N) of genes from male lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. All groups were compared by Kruskal-Wallis test (a: p = 0.0584; c: p = 0.0839; p > 0.1 in all other cases) followed by *post hoc* Dunn's tests where applicable (b: p = 0.0187; d: p = 0.0437; e: p = 0.0531). Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		
		U	N	O
Adipogenesis	C/EBPα	0.84 ± 0.20	1.00 ± 0.17	1.31 ± 0.36
	NR3C1	0.83 ± 0.08	1.00 ± 0.14	0.81 ± 0.09
	PPARγ	0.76 ± 0.18	1.00 ± 0.16	0.83 ± 0.10
	SREBF1	1.17 ± 0.26	1.00 ± 0.22	1.27 ± 0.35
Metabolism	ADIPOQ	0.59 ± 0.11 [a,b]	1.00 ± 0.13 [a,b]	0.77 ± 0.12 [a]
	FABP4	0.74 ± 0.09	1.00 ± 0.09	1.01 ± 0.13
	GPR120	0.77 ± 0.11	1.00 ± 0.16	0.60 ± 0.07
	INSR	0.71 ± 0.14	1.00 ± 0.19	1.06 ± 0.16
	LEP	0.89 ± 0.11	1.00 ± 0.12	0.85 ± 0.03
	RIP140	1.10 ± 0.20	1.00 ± 0.13	0.77 ± 0.10
Thermogenesis	ATF2	0.89 ± 0.11	1.00 ± 0.07	0.86 ± 0.09
	CIDEA	0.57 ± 0.13	1.00 ± 0.27	0.91 ± 0.25
	DIO2		Not quantifiable	
	PGC1α	2.02 ± 1.13 [c,d]	1.00 ± 0.31 [c,e]	0.25 ± 0.02 [c,d,e]
	PRLR		Not quantifiable	
	UCP1		Not quantifiable	
Development	HOXC9	0.73 ± 0.13	1.00 ± 0.11	1.07 ± 0.04
	LHX8		Not quantifiable	
	PRDM16		Not quantifiable	
	SHOX2	1.03 ± 0.16	1.00 ± 0.10	1.15 ± 0.33

Table D-7 Gene expression of female lambs in sternal adipose tissue by prenatal feeding group. Feeding groups are: undernutrition (U: n = 5); overnutrition (O: n = 6). There were no females in the control group (N). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the undernutrition group (U) of genes from female lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (p > 0.1 in all cases). Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)	
		U	O
Adipogenesis	C/EBPα	1.00 ± 0.25	1.46 ± 0.38
	NR3C1	1.00 ± 0.20	0.85 ± 0.11
	PPARγ	1.00 ± 0.17	1.02 ± 0.10
	SREBF1	1.00 ± 0.22	1.17 ± 0.37
Metabolism	ADIPOQ	1.00 ± 0.16	1.19 ± 0.19
	FABP4	1.00 ± 0.11	1.06 ± 0.19
	GPR120	1.00 ± 0.30	1.55 ± 0.34
	INSR	1.00 ± 0.22	0.95 ± 0.16
	LEP	1.00 ± 0.18	0.81 ± 0.15
	RIP140	1.00 ± 0.12	0.77 ± 0.12
Thermogenesis	ATF2	1.00 ± 0.10	0.83 ± 0.09
	CIDEA	1.00 ± 0.34	0.76 ± 0.17
	DIO2	Not quantifiable	
	PGC1α	1.00 ± 0.30	0.73 ± 0.19
	PRLR	Not quantifiable	
	UCP1	Not quantifiable	
Development	HOXC9	1.00 ± 0.26	1.07 ± 0.18
	LHX8	Not quantifiable	
	PRDM16	Not quantifiable	
	SHOX2	1.00 ± 0.19	0.85 ± 0.18

Table D-8 Gene expression of male lambs in subcutaneous adipose tissue by prenatal feeding group. Feeding groups are: undernutrition (U: n = 4); 'normal' nutrition (N: n = 6); overnutrition (O: n = 4). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (N) of genes from male lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. All groups were compared by Kruskal-Wallis test (p > 0.1 in all cases). Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		
		U	N	O
Adipogenesis	C/EBPα	1.06 ± 0.18	1.00 ± 0.13	1.09 ± 0.25
	NR3C1	1.09 ± 0.16	1.00 ± 0.13	1.06 ± 0.19
	PPARγ	1.01 ± 0.23	1.00 ± 0.13	1.14 ± 0.23
	SREBF1	0.98 ± 0.16	1.00 ± 0.12	1.04 ± 0.10
Metabolism	ADIPOQ	1.04 ± 0.15	1.00 ± 0.11	1.21 ± 0.17
	FABP4	0.95 ± 0.17	1.00 ± 0.12	1.26 ± 0.26
	GPR120	1.61 ± 0.42	1.00 ± 0.34	1.53 ± 0.38
	INSR	0.82 ± 0.19	1.00 ± 0.16	1.26 ± 0.30
	LEP	0.97 ± 0.27	1.00 ± 0.31	1.18 ± 0.41
	RIP140	1.03 ± 0.29	1.00 ± 0.15	1.09 ± 0.24
Thermogenesis	ATF2	1.10 ± 0.14	1.00 ± 0.09	1.10 ± 0.10
	CIDEA	0.75 ± 0.08	1.00 ± 0.27	1.18 ± 0.53
	DIO2	Not quantifiable		
	PGC1α	3.16 ± 2.65	1.00 ± 0.48	0.60 ± 0.27
	PRLR	Not quantifiable		
	UCP1	Not quantifiable		
Development	HOXC9	0.88 ± 0.14	1.00 ± 0.23	1.23 ± 0.25
	LHX8	Not quantifiable		
	PRDM16	Not quantifiable		
	SHOX2	0.89 ± 0.30	1.00 ± 0.22	0.93 ± 0.30

Table D-9 Gene expression of female lambs in subcutaneous adipose tissue by prenatal feeding group. Feeding groups are: undernutrition (U: n = 5); overnutrition (O: n = 5/6). There were no females in the control group (N). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the undernutrition group (U) of genes from female lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test (p > 0.1 in all cases). Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)	
		U	O
Adipogenesis	C/EBPα	1.00 ± 0.07	1.33 ± 0.20
	NR3C1	1.00 ± 0.14	0.98 ± 0.10
	PPARγ	1.00 ± 0.17	1.12 ± 0.21
	SREBF1	1.00 ± 0.09	1.07 ± 0.10
Metabolism	ADIPOQ	1.00 ± 0.22	1.14 ± 0.24
	FABP4	1.00 ± 0.10	1.22 ± 0.13
	GPR120	1.00 ± 0.09	1.16 ± 0.29
	INSR	1.00 ± 0.20	1.21 ± 0.36
	LEP	1.00 ± 0.17	1.14 ± 0.22
	RIP140	1.00 ± 0.12	0.98 ± 0.19
Thermogenesis	ATF2	1.00 ± 0.11	1.11 ± 0.12
	CIDEA	1.00 ± 0.21	1.09 ± 0.29
	DIO2	Not quantifiable	
	PGC1α	1.00 ± 0.55	0.81 ± 0.30
	PRLR	Not quantifiable	
	UCP1	Not quantifiable	
Development	HOXC9	1.00 ± 0.26	0.90 ± 0.18
	LHX8	Not quantifiable	
	PRDM16	Not quantifiable	
	SHOX2	1.00 ± 0.27	0.96 ± 0.28

Table D-10 Gene expression of male lambs in sternal adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 7); high-carbohydrate, high-fat (HCHF) (H: n = 8). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from male lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test. NA is not applicable. Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		p
		C	H	
Adipogenesis	C/EBPα	1.00 ± 0.11	0.48 ± 0.08	0.0037
	NR3C1	1.00 ± 0.09	0.70 ± 0.05	0.0140
	PPARγ	1.00 ± 0.06	0.52 ± 0.06	0.0006
	SREBF1	1.00 ± 0.15	0.50 ± 0.03	0.0541
Metabolism	ADIPOQ	1.00 ± 0.09	0.58 ± 0.07	0.0093
	FABP4	1.00 ± 0.08	0.77 ± 0.08	0.0721
	GPR120	1.00 ± 0.12	1.13 ± 0.18	> 0.1000
	INSR	1.00 ± 0.11	0.60 ± 0.09	0.0401
	LEP	1.00 ± 0.06	1.10 ± 0.12	> 0.1000
	RIP140	1.00 ± 0.13	0.88 ± 0.11	> 0.1000
Thermogenesis	ATF2	1.00 ± 0.09	1.09 ± 0.08	> 0.1000
	CIDEA	1.00 ± 0.19	0.47 ± 0.06	0.0401
	DIO2	Not quantifiable		NA
	PGC1α	1.00 ± 0.25	1.71 ± 0.92	> 0.1000
	PRLR	Not quantifiable		NA
	UCP1	Not quantifiable		NA
Development	HOXC9	1.00 ± 0.09	1.04 ± 0.13	> 0.1000
	LHX8	Not quantifiable		NA
	PRDM16	Not quantifiable		NA
	SHOX2	1.00 ± 0.09	0.61 ± 0.08	0.0059

Table D-11 Gene expression of female lambs in sternal adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 6); high-carbohydrate, high-fat (HCHF) (H: n = 5). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from female lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test. NA is not applicable. Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		p
		C	H	
Adipogenesis	C/EBPα	1.00 ± 0.19	0.44 ± 0.11	0.0303
	NR3C1	1.00 ± 0.07	0.51 ± 0.07	0.0043
	PPARγ	1.00 ± 0.03	0.58 ± 0.06	0.0043
	SREBF1	1.00 ± 0.19	0.45 ± 0.16	0.0823
Metabolism	ADIPOQ	1.00 ± 0.01	0.72 ± 0.21	> 0.1000
	FABP4	1.00 ± 0.08	1.08 ± 0.24	> 0.1000
	GPR120	1.00 ± 0.14	1.53 ± 0.45	> 0.1000
	INSR	1.00 ± 0.08	0.50 ± 0.11	0.0043
	LEP	1.00 ± 0.20	0.80 ± 0.09	> 0.1000
	RIP140	1.00 ± 0.12	0.81 ± 0.14	> 0.1000
Thermogenesis	ATF2	1.00 ± 0.12	0.90 ± 0.07	> 0.1000
	CIDEA	1.00 ± 0.17	0.74 ± 0.35	> 0.1000
	DIO2	Not quantifiable		NA
	PGC1α	1.00 ± 0.15	0.31 ± 0.02	0.0043
	PRLR	Not quantifiable		NA
	UCP1	Not quantifiable		NA
Development	HOXC9	1.00 ± 0.20	0.94 ± 0.21	> 0.1000
	LHX8	Not quantifiable		NA
	PRDM16	Not quantifiable		NA
	SHOX2	1.00 ± 0.08	0.43 ± 0.08	0.0043

Table D-12 Gene expression of male lambs in subcutaneous adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 7); high-carbohydrate, high-fat (HCHF) (H: n = 7). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from male lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test. NA is not applicable. Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		p
		C	H	
Adipogenesis	C/EBPα	1.00 ± 0.08	0.63 ± 0.09	0.0070
	NR3C1	1.00 ± 0.07	0.65 ± 0.05	0.0023
	PPARγ	1.00 ± 0.08	0.60 ± 0.07	0.0041
	SREBF1	1.00 ± 0.06	0.71 ± 0.07	0.0175
Metabolism	ADIPOQ	1.00 ± 0.07	0.73 ± 0.08	0.0262
	FABP4	1.00 ± 0.07	0.87 ± 0.16	> 0.1000
	GPR120	1.00 ± 0.17	1.48 ± 0.36	> 0.1000
	INSR	1.00 ± 0.12	0.58 ± 0.09	0.0111
	LEP	1.00 ± 0.15	2.69 ± 0.42	0.0175
	RIP140	1.00 ± 0.07	0.51 ± 0.08	0.0023
Thermogenesis	ATF2	1.00 ± 0.07	0.82 ± 0.06	0.0728
	CIDEA	1.00 ± 0.24	0.48 ± 0.06	0.0728
	DIO2	Not quantifiable		NA
	PGC1α	1.00 ± 0.59	0.19 ± 0.06	0.0379
	PRLR	Not quantifiable		NA
	UCP1	Not quantifiable		NA
Development	HOXC9	1.00 ± 0.11	0.54 ± 0.10	0.0111
	LHX8	Not quantifiable		NA
	PRDM16	Not quantifiable		NA
	SHOX2	1.00 ± 0.14	0.51 ± 0.13	0.0175

Table D-13 Gene expression of female lambs in subcutaneous adipose tissue by postnatal feeding group. Feeding groups are: control (C: n = 5/6); high-carbohydrate, high-fat (HCHF) (H: n = 5). Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from female lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes PGK1, SDHA, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test. NA is not applicable. Expression of five genes was too low for reliable quantification.

Category	Gene	Relative expression (AU)		p
		C	H	
Adipogenesis	C/EBPα	1.00 ± 0.16	0.81 ± 0.08	> 0.1000
	NR3C1	1.00 ± 0.04	0.63 ± 0.04	0.0079
	PPARγ	1.00 ± 0.08	0.48 ± 0.05	0.0043
	SREBF1	1.00 ± 0.06	0.74 ± 0.03	0.0079
Metabolism	ADIPOQ	1.00 ± 0.07	0.40 ± 0.08	0.0043
	FABP4	1.00 ± 0.05	0.74 ± 0.11	> 0.1000
	GPR120	1.00 ± 0.26	0.92 ± 0.10	> 0.1000
	INSR	1.00 ± 0.16	0.41 ± 0.06	0.0079
	LEP	1.00 ± 0.24	1.28 ± 0.14	> 0.1000
	RIP140	1.00 ± 0.12	0.60 ± 0.04	0.0173
Thermogenesis	ATF2	1.00 ± 0.09	0.77 ± 0.07	0.0952
	CIDEA	1.00 ± 0.20	0.55 ± 0.10	> 0.1000
	DIO2	Not quantifiable		NA
	PGC1α	1.00 ± 0.33	0.26 ± 0.07	0.0823
	PRLR	Not quantifiable		NA
	UCP1	Not quantifiable		NA
Development	HOXC9	1.00 ± 0.08	0.43 ± 0.13	0.0043
	LHX8	Not quantifiable		NA
	PRDM16	Not quantifiable		NA
	SHOX2	1.00 ± 0.30	0.76 ± 0.16	> 0.1000

Table D-14 Gene expression of male lambs by postnatal feeding group: effect of outlier removal. Feeding groups are: control (C: n = 7); high-carbohydrate, high-fat (HCHF) (H: n = 7); n is shown before removal of the outlier; M is male, F is female. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from male lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ (sternal) or PGK1, SDHA, TBP and YWHAZ (subcutaneous). The two groups were compared by Mann-Whitney U-test.

Tissue	Gene	Outl	Complete			Minus outlier		
			Rel expression (AU)		p	Rel expression (AU)		p
			C	H		C	H	
Sternal	PGC1α	H	1.00 ± 0.25	1.71 ± 0.92	> 0.1000	1.00 ± 0.25	0.84 ± 0.36	> 0.1000
Subcutaneous	FABP4	H	1.00 ± 0.07	0.87 ± 0.16	> 0.1000	1.00 ± 0.07	0.72 ± 0.07	0.0350
	PGC1α	C	1.00 ± 0.59	0.19 ± 0.06	0.0379	1.00 ± 0.44	0.45 ± 0.13	0.0734

Table D-15 Gene expression of female lambs by postnatal feeding group: effect of outlier removal. Feeding groups are: control (C: n = 5/6); high-carbohydrate, high-fat (HCHF) (H: n = 5); n is shown before removal of the outlier; M is male, F is female. The entries in the Outl(ier) column marked with a superscript 'a' are the same animal. Values shown are mean expression in arbitrary units (AU) ± SEM relative to the control group (C) of genes from female lambs sampled at 6 months. Expression of genes of interest was normalised to the geometric mean expression of reference genes IPO8, TBP and YWHAZ. The two groups were compared by Mann-Whitney U-test.

Tissue	Gene	Outl	Complete			Minus outlier		
			Rel expression (AU)		p	Rel expression (AU)		p
			C	H		C	H	
Sternal	ADIPOQ	H [a]	1.00 ± 0.01	0.72 ± 0.21	> 0.1000	1.00 ± 0.01	0.51 ± 0.05	0.0095
	FABP4	H [a]	1.00 ± 0.08	1.08 ± 0.24	> 0.1000	1.00 ± 0.08	0.85 ± 0.09	> 0.1000
	CIDEA	H	1.00 ± 0.17	0.74 ± 0.35	> 0.1000	1.00 ± 0.17	0.42 ± 0.16	0.0667

E Papers and conference presentations

E.1 Papers published

Khanal P, Axel AMD, Kongsted AH, Husted SV, Johnsen L, Pandey D, Pedersen KL, Birtwistle M, Markussen B, Kadarmideen HN *et al* (2015), Late gestation under- and overnutrition have differential impacts when combined with a post-natal obesogenic diet on glucose-lactate-insulin adaptations during metabolic challenges in adolescent sheep, *Acta Physiol*, **213**(2), 519-536.

Ojha S, Birtwistle M, Budge H and Symonds ME (2013), Brown adipose tissue: a new human organ?, *Expert Rev Endocrinol Metab*, **8**(2), 123-125. (Review of a themed meeting of The Physiological Society, 'Brown adipose tissue: a new human organ?', London, UK, 2012.)

E.2 Conference presentations

Birtwistle M, Khanal P, Kongsted AH, Nielsen MO, Budge H and Symonds ME (2014), The impact of high energy intake during early postnatal life on gene regulators for thermogenesis, adipogenesis and metabolism in the sternal and subcutaneous adipose tissue depots of sheep, *Proc Physiol Soc*, **32**, abstract no PC027. Poster presented at a themed meeting of The Physiological Society, 'Obesity – a physiological perspective', Newcastle, UK, September 2014.

Birtwistle M, Khanal P, Kongsted A, Nielsen M, Budge H and Symonds ME (2013), Impact of increased carbohydrate and fat intake during postnatal and juvenile life on gene expression in the sternal fat of sheep, *Proc 37th IUPS*, abstract no PCD284. Poster presented at the 37th IUPS Conference, Birmingham, UK, July 2013.

Pope M, Birtwistle M, Budge H, Symonds ME (2013), Clavicular brown adipose tissue in the sheep has a different origin from perirenal brown adipose tissue that is indicative of a muscle related lineage, *Acta Obstet Gynecol Scand*, **92** (Suppl 160), abstract no PS33. Poster presented (by the author) at the Joint Symposium of the Danish Centre For Fetal Programming and the EU FP7 EarlyNutrition Consortium, Copenhagen, March 2013.