The influence of maternal diet during lactation on milk fatty acid profile and offspring's adipose tissue thermogenic capacity

By

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Abstract

Over the last two decades, obesity has become a major public health concern. Evidence suggests that obesity can be programmed by nutrition in early life.

A way to treat/prevent obesity would be a major breakthrough for public health and brown adipose tissue (BAT) has been identified as a potential target for this, due to its propensity to utilise stored energy to produce heat, a process mediated by the uncoupling protein (UCP) 1 gene. In humans and sheep, UCP1 decreases with age. Diet during lactation and subsequent alterations in milk fatty acid profile have not been studied in relation to effects on offspring BAT function. There is evidence from *in vitro* and rodent studies to suggest the potential for some fatty acids, such as conjugated linoleic acid (CLA), to increase UCP1 expression.

The aim of this thesis is to use sheep as a model to investigate the potential to alter the milk fatty acid profile by supplementing ewes' diet with canola or sunflower oil, as previous research has found these to be effective in increasing milk CLA. I then investigated the effects on offspring in early life, with a particular focus on their BAT and its UCP1 abundance. The perirenal adipose depot was studied due to it being the largest depot in young sheep, which is known to transition from brown to white adipose tissue in the first month of life.

In addition to this, I set out to investigate the CLA concentrations of infant formula milk and how they compare to those reported for human breast milk.

From the day of delivery, ewes raising two lambs each were supplemented with 3% canola or sunflower oil or fed a control diet. Milk samples and ewe and lamb weights were taken at 7 and 28 days and offspring were tissue and blood sampled at either 7 or 28 days. The mRNA expression several of genes associated with thermogenesis was measured.

Results showed that supplementation of ewe's diet with canola, and to a lesser extent, sunflower oil, resulted in decreases in milk medium-chain saturated fatty acids (SFAs). Both supplements increased milk monounsaturated fatty acids (MUFAs) by ~50%, and total and *cis*-9, *tran*s-11 CLA by ~80%. In addition, sunflower supplementation increased omega-6 PUFAs by 25% and the *trans*-10, *cis*-12 CLA isomer by 177% at 28 days of lactation compared to controls. Female lambs fed by sunflower oil supplemented ewes showed a 50% increase in adipose tissue weights compared with controls. Male and, to a lesser extent, female lambs fed by canola oil supplemented ewes showed increased UCP1 abundance at 28 days of age compared with controls. There was an increase in mRNA

expression of other thermogenic genes in the canola females at 7 but not 28 days, however, not in males, so this unlikely to be the main cause of the increases seen in UCP1. Results from my investigation into infant formula showed that CLA was present in 67% of the infant formula milks tested but at lower concentrations to those reported in human breast milk.

In conclusion, it is possible to induce changes in ewes' milk fatty acid profile with dietary supplementation and I have shown that the fatty acid profile of milk received in early life has the potential to affect adipose tissue development and UCP1 gene expression. If in the prevention of the normal decline in UCP1 were to be maintained into adulthood it could improve long-term health by helping to maintain body weight. Further research is now necessary to establish whether this increase in UCP1 is maintained beyond the sampling time points of my investigation.

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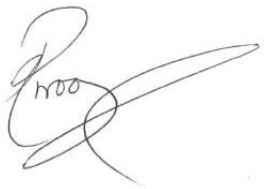
Declaration

The work in this thesis was performed within the Academic Division of Child Health, Obstetrics and Gynaecology at Nottingham University Hospital, Queens Medical Centre, Nottingham, between October 2011 and September 2016. I spent some time in 2015 working on an industrial placement as part of my BBSRC Industrial CASE Partnership with Danone Nutricia Research and Development Centre in Utrecht, The Netherlands.

Except where otherwise stated, this thesis represents my own work, carried out under the supervision of Professor Michael Symonds, Professor Helen Budge of the University of Nottingham and Dr Frank Wiens of Danone.

This thesis is an accurate representation of my work and, to my knowledge, no other study has been performed reproducing this work at the University of Nottingham.



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Conference Presentations

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Oral presentations

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Woods RM, Birtwistle MDA, Davies G, Perry V, Budge H, Stahl B, Wiens F & Symonds M. Supplementation of the maternal diet with canola oil during lactation leads to increased UCP1 levels in adipose tissue of the offspring. *The Physiological Society's Physiology 2015, Cardiff, UK.* July 2015.*

Woods RM, Birtwistle MDA, Davies G, Perry V, Budge H, Wiens F & Symonds M. Maternal fatty acid supplementation and its effects on milk composition and offspring body composition. *University of Nottingham School of Medicine's Sue Watson Postgraduate Presentations.* May 2014. **

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Woods RM, Birtwistle MDA, Davies G, Perry V, Budge H, Wiens F & Symonds M. Maternal dietary supplementation of fatty acids and its effects on milk composition and offspring adipose tissue development. *Obesity: A Physiological Perspective (Topic Meeting of the Physiological Society), Newcastle, UK*. September 2014.

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AA	Arachidonic acid
ACC	Acetyl-coenzyme a carboxylase
Acetyl-CoA	Acetyl-coenzyme a
ADRB3	Beta-3 adrenergic receptor
APS	Ammonium persulphate
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCA	Bicinchoninic Acid
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic protein
BMR	Basal metabolic rate
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
CIDEA	Cell death-inducing DNA fragmentation factor
CLA	Conjugated linoleic acid
CREB	cAMP response element-binding protein
CT	Cycle threshold
CVD	Cardiovascular disease
DAB	3,3'-Diaminobenzidine
DEXA	Dual energy X-ray absorptiometry
DHA	Docosahexaenoic acid
DIO2	Type 2 iodothyronine deiodinase
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FABP4	Fatty acid binding protein 4

FAS Fatty acid synthase

FBS Fetal bovine serum

FDG 18F-fludeoxyglucose

FFA Free fatty acids

FO Follow on formula

gDNA Genomic deoxyribonucleic acid

GGT Gamma-glutamyl transpeptidase

GR Glucocorticoid receptor

GUM Growing up milk

H&E Haematoxylin and eosin

HMF Human milk fat

HOXC9 Homeobox C9

HPLC High-performance liquid chromatography

HRP Horseradish peroxidase

HSL Hormone sensitive lipase

IBMX 3-isobutyl-1-methylxanthine

IF Infant formula

IHC Immunohistochemistry

KLFs Kruppel-like factors

LA Linoleic acid

LCFA Long-chain fatty acid

LCPUFA Long-chain polyunsaturated fatty acid

LNA α-linolenic acid

LPL Lipoprotein lipase

MRI Magnetic resonance imaging

MSCs Mesenchymal stem cells

MUFA Monounsaturated fatty acid

MYF5 Myogenic factor 5

NADH Reduced nicotinamide adenine dinucleotide

NCD Noncommunicable disease

NEFA Non-esterified fatty acids

NHS National health service

NRTC No reverse transcriptase control

NST Non-shivering thermogenesis

NTC	No template control
PCR	Polymerase chain reaction
PET	positron emission tomography
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PRDM16	PR domain containing
PRLR	Prolactin receptor
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative polymerase chain reaction
RIP140	Receptor-interacting protein 140
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEC	Socio-economic class
SEM	Standard error of mean
SFA	Saturated fatty acid
SHOX2	Short stature homeobox 2
SNS	Sympathetic nervous system
T3	Triiodothyronine
T4	Thyroxine
TAE	Tris-acetate ethylenediaminetetraacetic acid
TCF21	Transcription factor 21
TEMED	Tetramethylethylenediamine
TFAs	Trans fatty acid
Tm	Melting temperature
TR	Thyroid hormone receptor
Tris	Tris (hydroxymethyl) aminomethane base
tRNA	transfer RNA
TTBS	Tris buffered saline with Tween®
UCP1	Uncoupling protein 1
UV	Ultraviolet
WAT	White adipose tissue
WHO	World health organization

1 Introduction

1.1 Overweight and obesity

1.1.1 Definition

In adults, body mass index (BMI) is used to determine overweight and obesity. This is calculated with the equation: $\text{BMI} = \frac{\text{Weight (kg)}}{(\text{Height (m)})^2}$.

Overweight is defined by the World Health Organization (WHO) as a BMI between 25.0 and 29.9 and obesity is defined as a BMI of 30.0 or more[1].

For children, the Health Survey for England uses the UK 1990 BMI charts[2] which compare the weight and height of a given child to the average weight and height of a healthy child. Children with BMIs over the 85th centile are classified as overweight and those with BMIs over the 95th centile are classified as obese.

The WHO uses a similar classification system, using the 2007 WHO growth reference growth charts[3]. Children aged ≥5 years of age are classified as overweight if they have a BMI for age greater than 1 standard deviation above the WHO Growth Reference median. Obesity is classified as a BMI for age greater than 2 standard deviations above the WHO Growth Reference median. Children under the age of 5 are classified as overweight or obese with a BMI for age over 2 and 3 standard deviations (respectively) above the WHO Growth Standards median.

1.1.2 Prevalence

1.1.2.1 Europe

In 2013, 33% of women and 41% of men, in the UK, were overweight (BMI of 25-29.9kg/m^2), whilst 24% of women and 26% of men were classified as obese[4] (BMI ≥30kg/m^2).

In the same year, amongst children aged 2-15 years, 16% of boys and 15% of girls were classed as obese (BMI <95th centile)[4]. When combined, the prevalence of overweight (BMI <85th centile) and obesity was 30% for boys and 29% for girls[4].

Childhood obesity in the UK peaked in around 2004 when 18% of boys and 19% of girls were classed as obese. However, the prevalence seems to have plateaued now, with no significant changes between 2009 and 2013[4].

Across the WHO European region, figures from 2009-10 show that 50% of adults are overweight (BMI of 25-29.9kg/m^2) or obese(BMI ≥30kg/m^2)[5] and it is estimated that amongst children aged 6-9 years, on average, 1 in 3 are overweight (>1 standard deviation above the WHO Growth Reference median) or obese (>2 standard deviations above the WHO Growth Reference median)[5].

1.1.2.2 Worldwide

The prevalence of obesity worldwide has nearly doubled between 1980 and 2008[6]. Amongst adults (≥18 years of age), in 2014, 39% were estimated to be overweight and 13% obese. In the same year, for children under 5, it was estimated that 41 million are overweight or obese worldwide[6].

Whilst undernutrition is still a large problem in many parts of the world, in 2016 the noncommunicable disease (NCD) Risk Factor Collaboration reported that more of the world's population is now obese than underweight[7].

1.1.3 Effects on health

Not only does childhood obesity result in short-term health risks, such as elevated blood pressure[8,9], raised total cholesterol[9] and impaired glucose tolerance[10], but those who are overweight or obese in childhood are at a higher risk of being so in adulthood[11]. In addition to this, the persistence of overweight into later life is greater with increasing levels of overweight[12-14] and increasing age[15-18].

This demonstrates why it is imperative that overweight and obesity are tackled effectively, or better still, prevented entirely in childhood.

In adulthood, there are numerous health implications associated with obesity, both directly and when combined with other diseases[19]. In the Western world obesity is now one of the biggest causes of ill health[20]. Health risks associated with increasing BMI include type 2 diabetes[21-25], cardiovascular disease (CVD)[26-29], stroke[30-32], hypertension[33,34], some types of cancer[35], osteoarthritis[36,37] and liver disease[34].

Aside from the negative physiological effects of obesity, it can also have a detrimental effect on an individual's mental health, with obesity being associated with increases in lifetime diagnoses of major depression and bipolar disorder[38]. Obese individuals may face stigmatisation and discrimination in various aspects of life, from the workplace to personal

relationships[39]. In childhood, those who are overweight or obese have more time off school[40,41] and are more likely to experience bullying[42].

1.1.4 Financial burden in the UK

With the increase in health complications comes an increase in the financial burden of obesity on the National Health Service (NHS) in the UK. The direct cost of overweight and obesity to the NHS in 2006-7 was £5.1 billion[43]. The indirect costs are estimated at up to £27 billion[44]. When combining this with the cost of obesity-related medication[45] and social care[46], alongside estimates of obesity-related sick days[47], the total annual cost of obesity in the UK is estimated to be £47 billion[46]. These figures demonstrate the clear financial incentives to develop effective ways to prevent and treat obesity.

1.1.5 Causes of obesity

On the most basic level, overweight and obesity are caused by a positive energy balance; i.e. when an individual's energy intake exceeds their energy expenditure. However, the aetiology of obesity is complex. Genetic[48,49], environmental[50] and social[51] factors all play a role, in varying degrees, in an individual becoming overweight or obese[52]. In order to tackle obesity on the public health scale needed, a deeper understanding of the underlying mechanisms which are involved is required, to help formulate ways in which these can be manipulated in order to improve public health.

1.2 Adipose tissue

1.2.1 Introduction

When conducting research into the mechanisms of obesity, adipose tissue is of particular interest. Excess adipose tissue is one of the primary outcomes of obesity and the WHO describe overweight and obesity as "abnormal or excessive fat accumulation that may impair health"[53].

Adipose tissue is a type of loose connective tissue, the main role of which is to store energy in the form of triglycerides (Section 1.3.4, Page 18). It is primarily composed of adipocytes (Figure 1.1), which in mammals, have been traditionally divided into two main classes: brown and white. However, recent evidence has emerged of a new type of adipocyte, named 'brite' (for 'brown-in-white') or 'beige'.

A sustained positive energy balance causes adipose tissue to expand, either by hypertrophy or hyperplasia. Hypertrophy is the term for the increase in the size of existing adipocytes as they take up more triglycerides, and adipocyte expansion is the most important mechanism for increased adiposity in adults[54,55]. Fat mass and adipocyte volume are correlated[56], and the percentage of an individual's body weight which is adipose tissue can expand from 2-3% up to 60-70% as a result of sustained positive energy balance[57]. Prolonged hypertrophy appears to be involved with promoting inflammation[58].

Hyperplasia is the term for the formation of new adipocytes, which is a major factor in adipose tissue growth in childhood[59], and adipocyte number continues to change in adolescence[60]. In adulthood, the number of adipocytes remains constant regardless of weight loss or gain[56]. However, adipocyte number is higher in obese compared to lean individuals[56], suggesting that adipocyte number in adulthood is determined in childhood, and is a major determinant of future obesity risk[56,61].

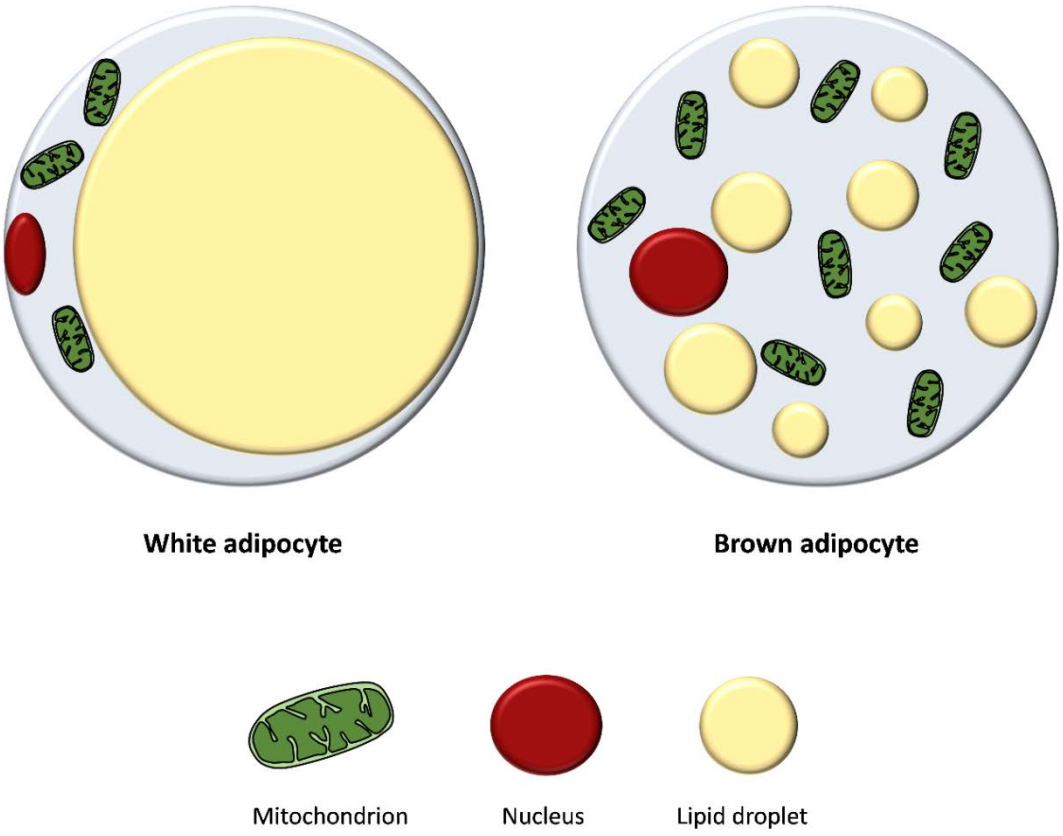

Figure 1.1 Schematic diagram of white and brown adipocytes.

White adipocytes consist of a singular lipid droplet (unilocular) surrounded by cytoplasm containing the nucleus and a small number of mitochondria. They range in diameter from approximately 10-200µm. Brown adipocytes have multiple lipid droplets (multilocular) containing the nucleus and an abundance of mitochondria within the cytoplasm. They are smaller than white adipocytes, at approximately 15-60µm in diameter. Diagram not to scale.

1.2.2 White adipose tissue

The primary role of white adipose tissue (WAT) is to store energy for use in times of negative energy balance. In times of positive energy balance, the size of adipocytes increases and their volume can expand 2-3 fold[55,62]. In addition to energy storage, WAT has a signalling function, secreting molecules called adipokines, including leptin and adiponectin. WAT also plays a role in the inflammatory process[63] and in glucose homeostasis[64,65].

The structure of a white adipocyte is a globular cell containing a large lipid droplet, which can account for up to 90% of its volume[66], with other organelles such as the nucleus and mitochondria being found near the edge of the cell (Figure 1.1).

WAT is located in depots throughout the body. Subcutaneous adipose tissue is located under the skin in areas such as the abdomen, intramuscular adipose tissue is found between the muscles, and visceral adipose tissue is found around the internal organs. The amount and position of WAT in humans varies between individuals, with factors such as gender playing a role[67].

The *leptin* gene, also known as LEP or Ob (obese), transcribes leptin, a hormone which is primarily secreted by WAT[68]. It has been termed the 'satiety hormone' due to its action in the hypothalamus, primarily in the arcuate nucleus where it affects appetite. Leptin works via a negative feedback loop which maintains homeostatic control of adipose tissue mass. When fat mass falls, so do levels of circulating leptin, and the reduction in leptin stimulates appetite and suppresses energy expenditure. When fat mass increases, there is an accompanying rise in circulating leptin and this acts to suppress appetite. *Leptin*-deficient (ob/ob) mice have a vast increase in fat mass compared to wildtype controls[69]. However, their increased fat mass can be reversed by the administration of biosynthetic leptin[70-72]. In humans, a rare condition of *leptin* deficiency has been reported by Montague and Farooqi *et al*., who found that children with a homozygous frameshift mutation in the *leptin* gene exhibited extreme hyperphagia and severe early onset obesity[48]. Remarkably, when treated

with recombinant leptin via subcutaneous injection, children with *leptin* deficiency are able to reach a normal body weight as their extreme hyperphagia is rectified[73].

Another genetic marker of WAT, transcription factor (TCF) 21, encodes a transcription factor of the basic helix-loop-helix family and is expressed in a variety of tissues. Walden *et al.* found it to be an effective genetic marker for WAT, due to its high expression in WAT compared to brown and brite adipose tissue[74].

1.2.3 Brown adipose tissue

Although first described in 1551[75], only in the last century has the role of brown adipose tissue (BAT) been characterised. Its primary function is as a thermogenic organ and is essential for classical non-shivering thermogenesis. BAT is activated at birth, in order to maintain the body temperature of the neonate upon entering the cold extrauterine environment. Until fairly recently, in humans it was believed that BAT was only functional in neonates, however, it has now been identified in adults[76-80]. The structure of a brown adipocyte is shown in Figure 1.1.

In both newborn sheep and humans, BAT is laid down primarily in late gestation[81] and constitutes around 1-2% of birth weight[82,83].

1.2.3.1 Non-shivering thermogenesis

Non-shivering thermogenesis (NST) is the cold-induced heat production which is not associated with the muscle activity of shivering. BAT plays a key role in NST, maintaining body temperature in cold environments. This is mediated by an inner mitochondrial membrane protein present in BAT, called uncoupling protein (UCP) 1, which uncouples respiration from oxidative phosphorylation, causing the chemical energy produced by respiration to be released as heat rather than being utilised to synthesise adenosine triphosphate (ATP). UCP1 is only expressed in BAT and brite adipose tissue, and is the most commonly used genetic marker of BAT[84]. UCP1 in BAT is activated at birth for thermoregulation in the cold extra-uterine environment[85], and animal studies have shown that this is caused by an increase in sympathetic nerve activity, which results in an increase of glucose uptake in the brown adipocytes[84].

More recently, BAT has been identified in adult humans[78-80,86,87]. In 1996, using positron emission tomography (PET) scans, which allow visualisation of the uptake of glucose, using ^{18}F-fludeoxyglucose (FDG), Barrington and Maisey observed patterns of FDG uptake in the

neck region of several patients[88]. However, it was not until 2007 that Nedergaard *et al*. reviewed this evidence and identified the areas of glucose uptake as BAT[76]. Following this, experimental studies on healthy adults[79,80,86] and clinical scans performed on patients[78,87] have all positively identified BAT in the cervical and thoracal region (Figure 1.3). The experimental studies showed little metabolic activity in BAT in a thermoneutral environment, but when subjects were in a cold environment, increased glucose uptake was observed in a number of BAT depots in between 52%[77] and 96%[80] of subjects. It appears that gender may have an effect on BAT activity, with more FDG uptake (indicating increased BAT activity), being observed in women compared to men[78,87]. Seasonal effects have also been noted, with the number of scans showing BAT and the number of FDG positive depots both increasing in the winter months[87]. The use of radiolabeled glucose is somewhat of a limitation, due to fatty acid uptake being the primary substrate used in BAT[89], which will not be measured in these scans.

1.2.3.2 Activation of BAT

UCP1 is activated by a signalling cascade that starts with norepinephrine binding to the β_3 adrenergic receptor, triggering cyclic adenosine monophosphate (cAMP) pathways which induce the transcription of *UCP1* and other thermogenic genes, and instigate protein kinase A (PKA)-dependant lipolysis. Long-chain fatty acids (LCFA) released by lipolysis then bind to *UCP1* and activate it (Figure 1.2).

One important thermogenic gene is type 2 iodothyronine deiodinase (DIO2). This encodes for the enzyme of the same name, which is responsible for the conversion of the thyroid prohormone thyroxine (T_4) to the bioactive triiodothyronine (T_3)[90]. T_3 binds to and activates the thyroid hormone receptor (TR), stimulating the expression of *UCP1* and *DIO2*[91]. This means that DIO2 effectively promotes its own expression, in a positive feedback loop.

In addition to cold exposure, the expression of *UCP1* can be altered by hormones, including prolactin. The action of prolactin is mediated by the prolactin receptor (PRLR), a transmembrane receptor which interacts with prolactin (and also growth hormone and placental lactogen), and which is highly expressed in BAT[92,93]. In sheep, the loss of PRLR is highly correlated with the loss of UCP1 in early life[94], and the stage in gestation at which PRLR abundance peaks coincides with the time that UCP1 is first detected[85,92]. Administration of prolactin to pregnant rats results in the pups' BAT possessing fewer lipid droplets but increased UCP1[95], which suggests that prolactin enhances the development of

brown fat. Postnatally, administration of prolactin to young sheep has also shown a thermogenic effect[94].

Another gene involved with thermogenesis is cell death-inducing DNA fragmentation factor α-like effector (CIDE) A, which, in rodents, is highly expressed in BAT, where its pattern of expression mirrors *UCP1*, but it is not expressed in WAT[96]. However, CIDEA appears to negatively regulate *UCP1*, inhibiting thermogenesis[97], and mice deficient in *CIDEA* are lean and resistant to diet-induced obesity, due to a greater energy expenditure and depletion of fat stores, as a result of enhanced *UCP1* activity[97]. Furthermore, *CIDEA*'s effects appear to be species specific, as in humans it is expressed in WAT[98], and a single nucleotide polymorphism in the *CIDEA* gene is associated with obesity, potentially due to *CIDEA*'s suppression of basal metabolic rate (BMR)[99]. In sheep, *CIDEA*'s function is not well characterised, although its expression appears to mirror that of *UCP1*[100], as seen in rodents[97].

Other genes which have been associated with the suppression of *UCP1* are receptor-interacting protein (RIP) 140[101] and *adiponectin*[102]. *RIP140* knockout mice are lean, resistant to high-fat diet induced obesity, and have increased *UCP1* expression in WAT[101], suggesting that *RIP140* suppresses the browning of WAT. *Adiponectin* is negatively correlated with obesity in human adults[103] and children[104], yet in the newborn period, it is positively correlated with birth weight[105] and adiposity[106]. It has been shown to reduce energy expenditure in mice[107], potentially through the inhibition of *UCP1*[102]. In sheep, *adiponectin* expression increases with age, whilst *UCP1* expression decreases[100].

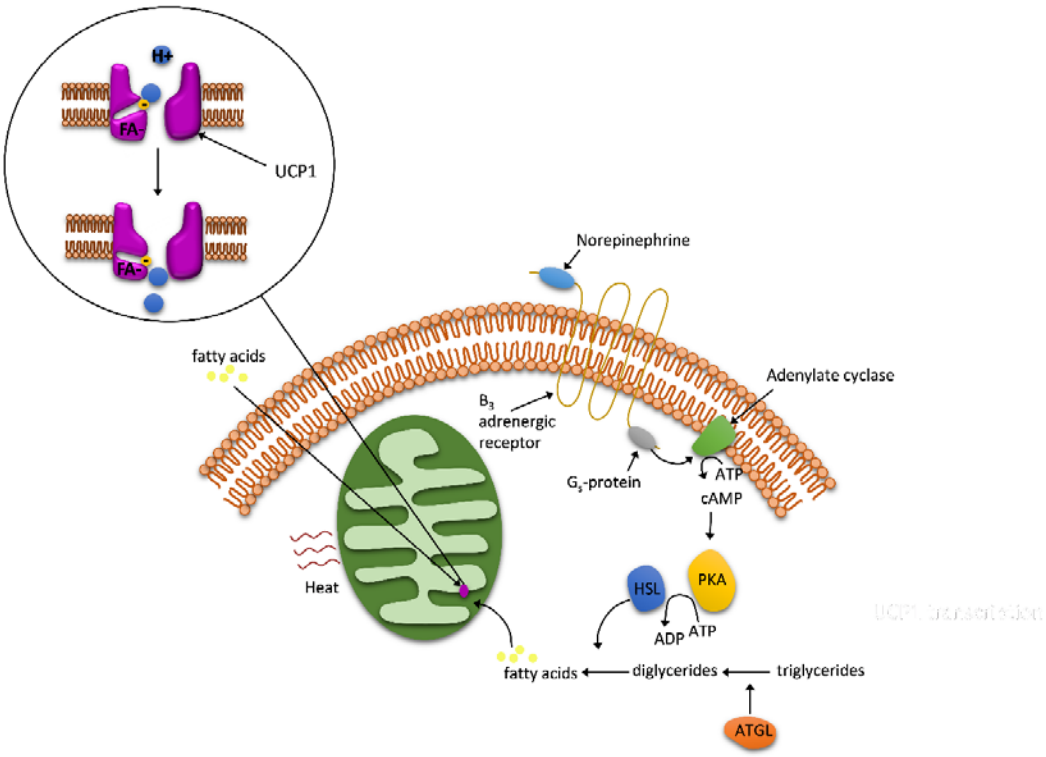

Figure 1.2 Illustration of the beta-3 adrenergic cascade which activates *UCP1*.

Upon cold exposure, norepinephrine binds with the β-3 adrenoceptor, activating adenylate cyclase which generates cyclic adenosine monophosphate (cAMP). This activates protein kinase A (PKA) which phosphorylates and activates hormone sensitive lipase (HSL). HSL accelerates the release of free fatty acids from triglyceride stores, a process which is catalysed by adipose triglyceride lipase (ATGL). Some of these free fatty acids activate uncoupling protein (UCP) 1, which uncouples the proton gradient in the inner mitochondrial membrane, resulting in energy being released in the form of heat. Diagram adapted from[108-110].

1.2.3.3 Anatomical locations of BAT

BAT is located in several depots throughout the body, which vary between species. The largest BAT depots also vary between species, with the interscapular depot being the largest in rodents[76] and the perirenal depot the largest in sheep[111]. In human adults, the main BAT depot is in the supraclavicular and neck areas[76], with other depots including those in the supra- and perirenal, thyroid/tracheal, mediastinal, and parathoracical anatomical locations[76,112] (Figure 1.3). Human infants also have a thin 'kite-shaped' layer of BAT in the interscapular region, however, this depot has been deemed to be quantitatively unimportant[113].

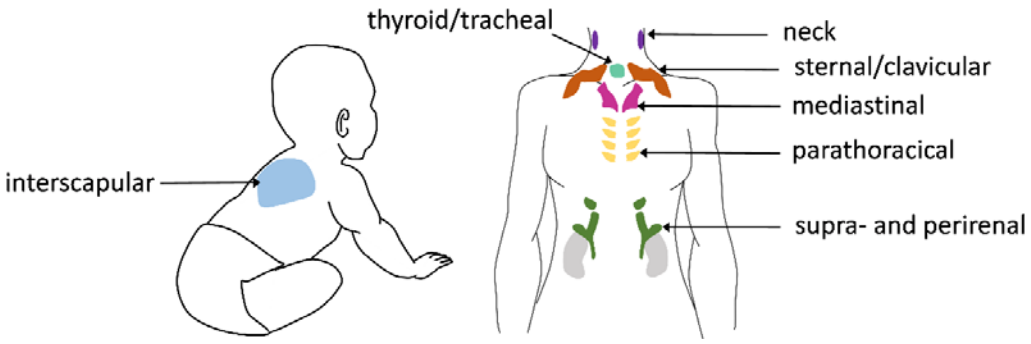

Figure 1.3 Anatomical locations of brown adipose tissue in human infants and adults.

Adults and infants possess common brown adipose tissue depots (shown on adult in diagram), with the exception of the interscapular depot, which is only present in infants (shown on infant in diagram). Diagram adapted from Enerbäck *et al*.[112].

1.2.4 Clinical relevance of BAT

From a public health perspective, BAT is of particular interest as unlike WAT, which has the primary function of storing energy, BAT expends energy by using stored energy for the generation of heat through the uncoupling process[84]. It has been estimated that up to 20% of total daily energy expenditure could be accounted for by 50g of BAT[114]. The effect which this could have over an extended period of time on an individual could be very useful in achieving or maintaining a healthy body weight.

If a way of increasing the activity of BAT, or preventing its decline into adulthood, was known, it could have a huge benefit on public health by leading to potential treatments for obesity and other metabolic disorders such as Type 2 Diabetes[115].

1.2.5 Potential risks of stimulating BAT

As previously described, BAT generates heat via the uncoupling process[84]. This in itself could potentially be harmful to individuals, if BAT was stimulated to the extent to which is raised core body temperature. The drug 2,4-Dinitrophenol (DNP) is sold as a weight loss drug, and appear to cause rapid weight loss, which has been suggested to be a result of increased uncoupling[116]. However, in 2011, in the medical literature DNP had been attributed to causing 62 deaths[117], with one of the mechanisms of toxicity being identified as hyperthermia, caused by the uncoupling of oxidative phosphyrlaton and associated dissipation of energy as heat[118].

It is unlikely that BAT would be stimulated to this extent by dietary interventions, however, there is potential for pharmacological interventions to run the risk of causing harm,

especially if the doses of drugs to stimulate BAT activity were calculated incorrectly/overestimated when translating rodent work to human intervention. These risks must be considered when conducting research into BAT stimulation, particularly if using pharmacological agents.

1.2.6 Brite adipose tissue

As previously mentioned, in addition to brown and white adipocytes, another type of adipocyte exists, known as 'brite' or 'beige'[119]. Brite adipocytes are brown-like adipocytes that were first discovered in rodents[120] and that can be induced in WAT depots by cold exposure or by beta-3 adrenergic receptor (ADRB3)/peroxisome proliferator-activated receptor (PPAR) γ agonists[120-127].

Brite adipocytes were originally believed to be classical brown adipocytes, but it has since been found that they are distinctive cells which have their own pattern of gene expression, expressing *UCP1*, but not some other brown specific genes[74]. They share many structural and biochemical characteristics with brown adipocytes, including the *ADRB3* pathway for regulation of thermogenic gene expression[128]. However, they originate from a different lineage to classical brown adipocytes, as discussed further on (Section 1.2.8.1, Page 12).

Perhaps most importantly of all, research in mice has shown that when stimulated, brite adipocytes have a similar thermogenic capacity to brown adipocytes[129,130]. This makes them attractive as potentially inducible adipocytes which could increase energy expenditure and therefore be used as a target for the prevention and treatment of obesity.

Homeobox (HOX) C9 and short stature homeobox (SHOX) 2 are genetic markers of brite adipose tissue. *HOXC9* was originally believed to be a WAT-specific gene[131], however, its expression has been found to be up-regulated in white adipocytes treated with rosiglitazone (a *PPARγ* agonist which is known to induce brite adipocytes)[131]. Further investigation has found that *HOXC9* is expressed in brite depots more highly than it is in white depots, and that it is hardly expressed at all in brown adipocytes[74]. This means that it serves as a useful gene to distinguish BAT from brite adipose tissue. *SHOX2* has been found to be almost exclusively expressed in brite adipose tissue compared to BAT and WAT[74].

1.2.7 Perirenal adipose tissue

The main focus of my investigations into offspring adipose tissue, in Chapters 3 and 5, is the perirenal adipose tissue depot. Using sheep as a model (discussed in Section 1.6, page 45).

This depot is found enveloping the kidneys and in human adults, brown adipocytes have been identified in the perirenal adipose tissue of 70-80% of participants studied[132,133]. In human infants, all perirenal adipose tissue samples contain high quantities of brown adipocytes. The number is lower in children and adolescents, reducing further in older adults[133]. The perirenal depot is estimated to account for around 75% - 80% of total adipose tissue in the neonatal sheep[83,111], and is brown at birth, undergoing a transition to WAT in the first month of life[134]. Using sheep as a model for my investigation allows me to focus on this 'critical window' in which their UCP1 usually declines, to determine whether nutrition in early life can prevent or reduce this delay.

1.2.8 Development of adipose tissue

1.2.8.1 Origin of adipocytes

Adipocytes are thought to derive from mesenchymal stem cells (MSCs), but the precise mechanism behind the commitment of MSCs to the adipocyte lineage is largely unknown[135]. It was previously believed that all adipocytes derive from MSCs from the mesoderm. However, recently it has been demonstrated that some adipocytes originate from the neuroectoderm[136].

It is now known that BAT and WAT develop from two distinct pathways. Classical brown adipocytes develop from myogenic factor (MYF) 5 expressing skeletal muscle precursors, by the action of PR domain containing (PRDM) 16[137], in association with CCAAT/enhancer-binding protein (C/EBP) β, which controls the 'switch' of cell fate from skeletal muscle cells to brown adipocytes[138]. Precursors of brite adipocytes are MYF5 negative, and more closely resemble those of white adipocytes[137], a recent study by Long *et al.* has shown that it is possible for brite adipocytes to arise from a smooth muscle-like origin[128]. Another study has found that the spectrum of adipocytes derived from MYF5+ precursors is larger than previously believed and that brown and white (and possibly brite) adipose tissues contain adipocyte progenitor cells from both MYF5+ and MYF5− lineages[139]. It is also possible for white adipocytes to undergo a cold-induced transdifferentiation to brite adipocytes[140].

1.2.8.2 Adipogenesis

Adipogenesis is a two-step process. The first step is the 'determination' of an MSC into a preadipocyte. The second step is the 'differentiation' of the preadipocyte into a lipid-filled adipocyte. Figure 1.4 illustrates the developmental pathways of white, brite and brown adipocytes.

Determination is the irreversible commitment of a cell to a particular pathway. Upon appropriate stimulation, pluripotent MSCs commit to the adipocyte lineage, and whilst the exact mechanisms are unknown, bone morphogenic protein (BMP) 2 and 4 and Wnt signalling are thought to be involved[141].

Differentiation has traditionally been studied using cell cultures with established preadipocyte lines. Differentiation is induced by a mixture of insulin, 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone[142]. Following induction, the preadipocyte undergoes several rounds of mitosis, after which it loses its fibroblast-like appearance, accumulates cytoplasmic triglycerides, and begins to acquire the morphology and metabolic features of an adipocyte[142,143], including the expression of adipogenic genes such as *leptin* and *adiponectin* (section 1.2.2, Page 12) and fatty acid binding protein (FABP) 4. *FABP4* is used as a marker of adipocyte differentiation[144] due to it being expressed very highly in mature adipocytes[145]. Its expression appears to be indicative of maturation of either BAT or WAT.

The hormonal induction of differentiation is followed by the activation of *C/EBPβ* and *C/EBPδ* which induce expression of the key regulators of adipocyte differentiation, *PPARγ* and *C/EBPα*[141]. PPARγ and C/EBPα are known to be positive regulators of *adiponectin*[146-148].

Several other transcription factors are involved in adipogenesis, including Kruppel-like factors (KLFs), glucocorticoid receptor (GR) and cAMP response element-binding protein (CREB), all of which are involved in regulating the expression and activity of *PPARγ* and *C/EBP*s. For brown fat adipogenesis, *PRDM16* is considered the 'master regulator of brown adipocytes'[149], as loss of PRDM16 from brown fat precursors results in a loss of brown fat characteristics and promotion of muscle differentiation, whereas ectopic expression of *PRDM16* in myoblasts induces differentiation into brown adipocytes[137]. PRDM16 acts by binding to PPARγ, activating its transcriptional function[137].

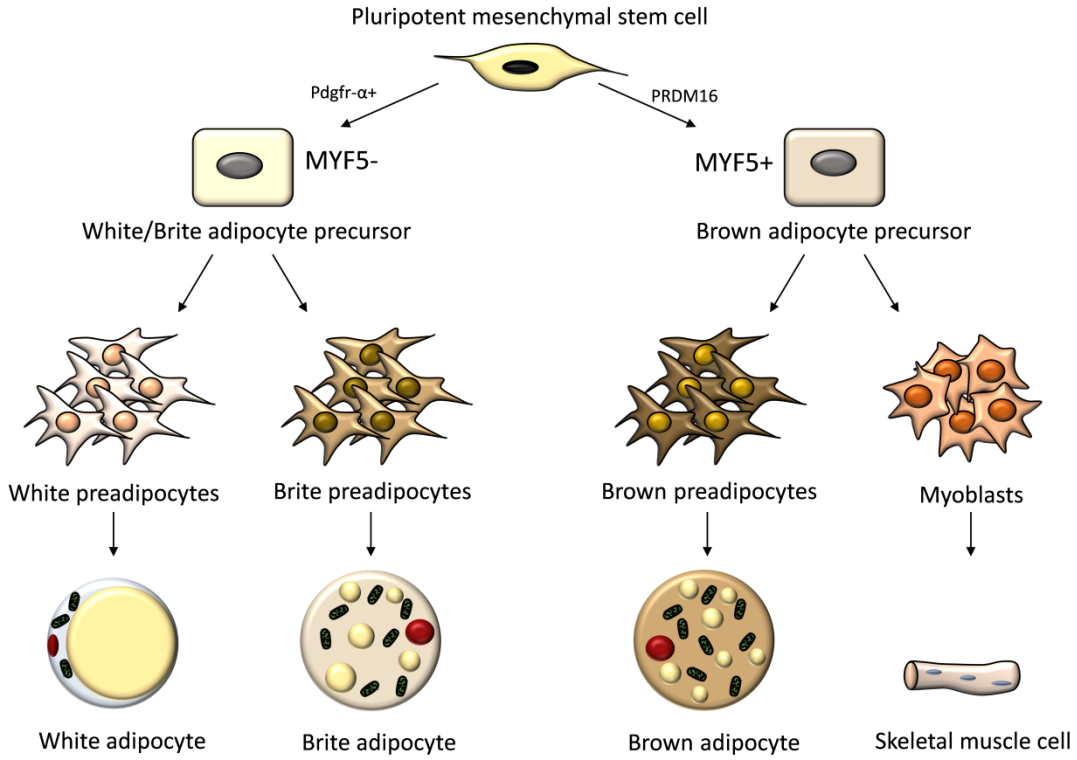

Figure 1.4 The developmental pathways of adipocytes.

White, brite and brown adipocytes all arise from pluripotent mesenchymal stem cells. Brown adipocytes are derived from myogenic factor (MYF) 5+ precursors and their brown adipocyte fate is determined by expression of *PRDM16*. White and brite adipocytes are derived from MYF5-, *Pdgfr-α+* expressing precursor cells. The exact lineage of adipocytes is not clearly understood, and it has recently emerged that a subset of brite adipocytes have a smooth muscle-like origin[128] (not illustrated). Diagram adapted from Reddy *et al.*[150].

1.3 Fatty acids

1.3.1 Introduction

Fatty acids are organic compounds that contain one or more carboxylic acid groups. Some fatty acids are 'saturated' – meaning that their carbon chain contains no double or triple bonds between the carbon atoms, and are therefore unable to incorporate any more hydrogen atoms (Figure 1.5). Other fatty acids are 'unsaturated' – meaning that their carbon chain possesses one or more double or triple bonds, and is, therefore, able to incorporate additional hydrogen atoms. An unsaturated fatty acid with one double bond is known as a monounsaturated fatty acid (MUFA; Figure 1.5) and those with two or more double bonds are known as polyunsaturated fatty acids (PUFAs; Figure 1.5).

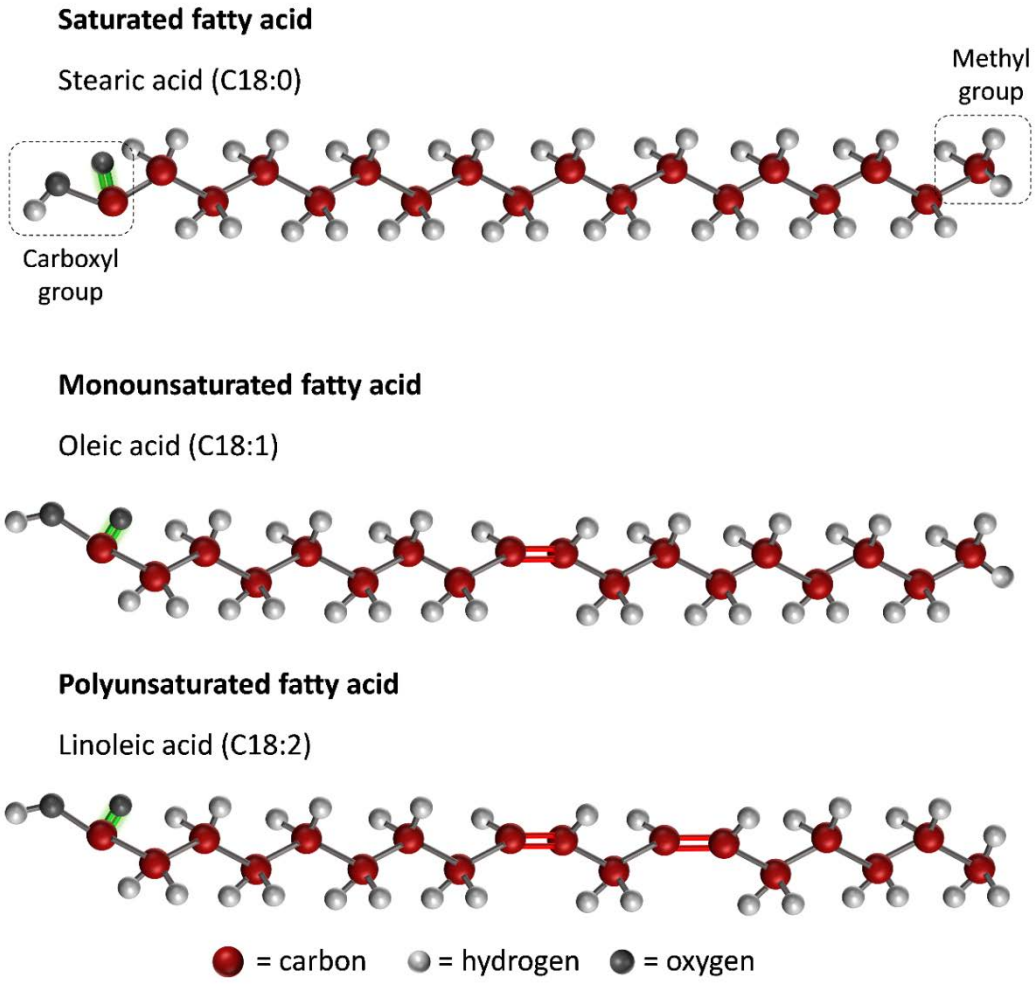

Figure 1.5 Structure of saturated, monounsaturated and polyunsaturated fatty acids.

The carbon chain of the saturated fatty acid has no double bonds and is therefore 'saturated' with hydrogen atoms. The unsaturated fatty acids have one (mono) or more (poly) carbon to carbon double bonds, and therefore do not have a full complement of hydrogen atoms.

Fatty acids are named in relation to the length of their carbon chain and the number of double bonds they contain. The number of carbons is written first, followed by a colon and then the number of double bonds. For example, linoleic acid (LA) has 18 carbons in its chain and contains two double bonds, so it is named C18:2. Those with 2-6 carbons are classed as short-chain fatty acids, medium-chain fatty acids contain 7-12 carbons, and fatty acids with more than 12 carbons are classed as LCFAs.

Fatty acids with the same number of carbon and hydrogen atoms can exist in different geometric forms, and these are called 'isomers'. When a fatty acid contains a carbon-

carbon double bond, there are two different configurations that the double bond may have – *cis* or *trans* (Figure 1.6). These refer to the position of the two parts of the main carbon chain in relation to the double bond, with the *cis* configuration having both parts of the carbon chain on the same side of the double bond, and the *trans* configuration having them on opposite sides. Different isomers of a fatty acid are identified by using the terms *cis*-n and *trans*-n, where n is the number of carbon atoms from the methyl end of the carbon chain to the start of the double bond.

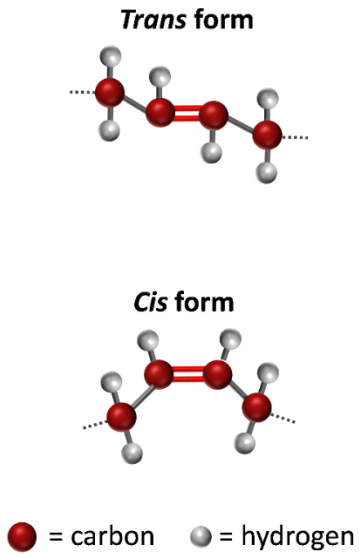

Figure 1.6 Illustration of the *cis* and *trans* configurations.

The *cis* form fatty acid appears 'bent' because the two parts of the carbon chain are on the same side of the double bond. The *trans* form fatty acid is straighter as the two parts of the carbon chain are on opposite sides of the double bond.

1.3.2 *Trans* fatty acids

Trans fatty acids (TFA) are unsaturated fatty acids which have at least one double bond in the *trans* configuration. They can arise in two ways: naturally, where they are synthesised in ruminant animals, or during the partial hydrogenation of vegetable oils – an industrial process which converts vegetable oils into semi-solid fats for use in food manufacturing. Therefore, the "*trans* fat" group can be divided into those found naturally in ruminant products (ruminant TFAs) and those occurring from partially hydrogenated vegetable oils, as a result of industrial processing (industrial TFAs).

The type of TFA is an important distinction which is being increasingly recognised. Several epidemiological studies have shown a clear link between industrial TFAs and CVD, but not between ruminant TFAs and CVD[151-157].

1.3.2.1 Legislation regulating TFAs in food

Dietary TFAs are generally associated with negative health outcomes, especially CVD[158,159], although as previously mentioned, this effect is likely to be caused by industrial, and not ruminant TFAs[151-157].

From a legislative viewpoint, the European Food Safety Authority (EFSA) do not currently make any distinction between ruminant and industrial TFAs, grouping them under the same "*trans* fat" category[160]. Legislation surrounding levels of TFAs in food varies between countries. Currently, the EFSA has set no specific limit on food TFA content[160], nor does the amount present have to be displayed on the food label[161]. Some countries have pioneered the introduction of TFA legislation. In 2003, Denmark became the first European country to introduce legislation regarding TFA levels in food, by stating that the TFA content in oils and fats used to produce foods must not exceed 2%[162]. It is difficult to judge the impact of this legislation on heart disease, which has fallen by approximately 70% in Denmark since 1980, before any TFA legislation was introduced, and is continuing to fall. Furthermore, the introduction of TFAs legislation has been accompanied by other health initiatives, including anti-smoking campaigning and the promotion of exercise and healthy eating. All of which, of course, are also concomitant with a decrease in the risk of heart disease[163-165].

Since Denmark introduced legislation, other countries, including Switzerland (in 2009), Austria (2009) and Iceland (2011), have followed suit with similar legislation[166]. In introducing this legislation, in all countries, a distinction between industrial and ruminant TFAs was made, and the legislation only applied to industrial TFAs. Similarly, in non-European countries, both the USA (2006) and Canada (2005) have laid down mandatory legislation concerning TFAs and made ruminant TFAs exempt from their compulsorily labelling rules[167]. Without making this exemption, common dairy products such as cheese and milk could be labelled as high in TFAs, due to ruminant TFAs. It is worthy to note that if ruminant TFAs were included, hypothetically, even breast milk could exceed the TFA threshold[168].

In the European Food and Nutrition Action Plan 2015-2020, the WHO laid out plans to "develop and implement national policies to ban or virtually eliminate *trans* fats from the food supply, with a view to making the European Region *trans* fat-free"[5]. When these plans are put into place, it seems very likely that a distinction will be made between industrial and ruminant TFAs in line with those countries which have already introduced legislation.

1.3.3 Essential fatty acids

Essential fatty acids are fatty acids which humans and other animals must obtain from dietary sources, due to the inability of the body to synthesise them. In humans these are α-linolenic acid (LNA, C18:3 n-3) and LA (C18:2 n-6). Mammals do not possess the desaturase enzymes to enable them to create double bonds closer to the methyl end of the chain than carbon 9. However, other omega-3 and -6 fatty acids can be synthesised in the body from LNA and LA[169].

1.3.4 Triglycerides

A triglyceride is an ester formed from a molecule of glycerol and three free fatty acids. This is the form in which lipids are stored within adipose tissue in times of positive energy balance. In times of negative energy balance, triglycerides are broken down, releasing fatty acids and glycerol into the circulation, thereby supplying energy to the body.

The position of fatty acids within a triglyceride has functional relevance. The positions are known as sn-1, sn-2 and sn-3. The sn-2 position (also known as the *beta* position) is central, and the sn-1 and sn-3 positions (collectively referred to as the *alpha* positions) are outside. The structure of a triglyceride is shown in Figure 1.7.

The positioning of the fatty acids within a triglyceride is particularly important in early life. For example, palmitic acid in human milk is generally esterified to the *beta* (sn-2) position[170,171], whilst in vegetable oils (often used as a fat source in infant formula milk[172,173]) it is generally in an *alpha* position[171]. In cows'[174,175] and sheep milk[176], palmitic acids position is about 50:50 between the *alpha* and *beta* positions. The *alpha* position is thought to be detrimental to intestinal fat and calcium absorption in the infant[177].

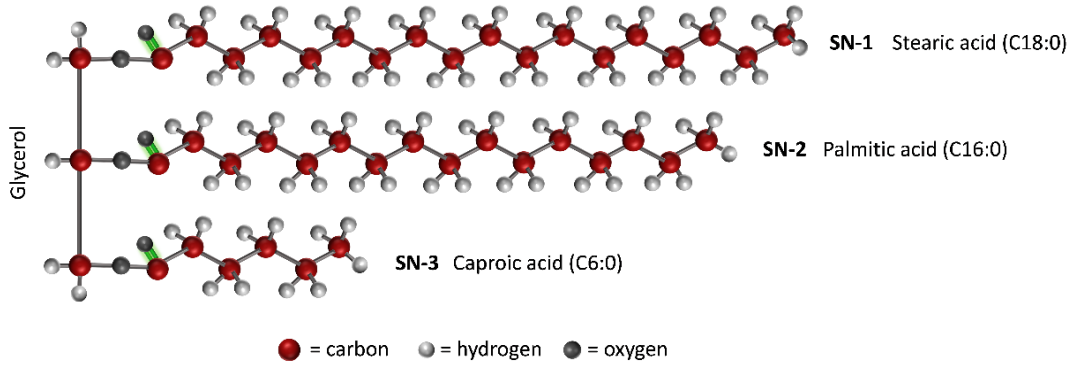

Figure 1.7 Example of the structure of a triglyceride.

Triglycerides are formed by a glycerol molecule with three hydroxyl groups, which bind to the carboxyl groups of three fatty acids, in this case stearic, palmitic and caproic acid. The sn- positions refer to the position of the individual fatty acid in relation to the others.

1.3.5 Biohydrogenation of fatty acids in the rumen

Like in human breast milk, in cow and sheep milk, approximately 98% of fat is in the form of triglycerides[178-180].

These are synthesised in the mammary epithelial cells from free fatty acids (FFAs) which can come from:

1) Lipids in the bloodstream (approximately 40-60%)
2) *De novo* synthesis in the mammary epithelial cells

Short- and medium-chain fatty acids tend to be derived from *de novo* synthesis, while LCFAs are usually absorbed from the bloodstream and are mainly derived from the diet[181,182]. Table 1.1 shows the percentages of fatty acids which are derived from *de novo* synthesis and blood lipids.

Fatty Acid	Derived from *de novo* synthesis (%)	Derived from blood lipids (%)
C4:0-C10:0	100	0
C12:0	80-90	10-20
C14:0	30-40	60-70
C16:0	20-30	70-80
C18:0	0	100

Table 1.1 Percentages of fatty acids derived from *de novo* synthesis and blood lipids. Based on values from Hurley[183].

1.3.6 The synthesis of milk fat in ruminants

Those fatty acids which are derived from lipids in the bloodstream ultimately originate from dietary sources. However, ruminants' diets are typically low in fat (1-2%)[184], and so the lipids are synthesised in the rumen, by bacteria. These bacteria separate the fatty acids and glycerol, and the glycerol is then fermented to volatile (short-chain) fatty acids.

Microbial lipases hydrolyse the ester linkages found in triglycerides, phospholipids and glycolipids when they first enter the rumen, resulting in the formation of non-esterified fatty acids (NEFAs). Unsaturated fatty acids will then be acted on by other bacteria in the rumen to produce saturated fatty acids (SFAs), this process is called biohydrogenation. In ruminants, over 90% of unsaturated fatty acids are biohydrogenated to SFAs[185]. In order to try to prevent this, ruminant protected fats have been developed, with the aim of protecting unsaturated fatty acids from ruminal hydrogenation and increasing the amount of unsaturated fatty acids present in the milk[186].

During the biohydrogenation process, intermediate compounds containing *trans*-double bonds are produced. Some of these intermediate products escape the rumen and are incorporated into tissue and milk fat, resulting in ruminant products containing TFAs[187].

Lipids leaving the rumen pass through the omasum and abomasum (the third and fourth part of a ruminant's stomach) rapidly, with little alteration to the composition. Microorganisms passing through are subjected to acid digestion and are broken down, causing them to release their fatty acids, which are then absorbed in the small intestine. When entering the duodenum, some fatty acids, such as those which are rumen-protected, are still unhydrolysed triglycerides. Pancreatic lipases act upon these, breaking them down into NEFAs and glycerol, which are then absorbed through the intestinal cells. The most abundant fatty acid for absorption is stearic acid, which is mainly converted to oleic acid by desaturases. After the absorption of fatty acids, they are reformed into triglycerides, by combining with glycerol, which is produced from the metabolism of glucose in the bloodstream. These triglycerides are then packed into lipoproteins, which enter the bloodstream and are delivered to organs, such as the mammary glands, where they are broken down into FFAs again by the enzyme lipoprotein lipase (LPL), which is present in the capillaries. The FFAs then enter the cells of the mammary gland and are formed back into triglycerides in the milk fat[185].

Fatty acids are synthesised *de novo* from acetate and 3-hydroxybutyrate in the cytoplasm of the mammary epithelial cells. The two key enzymes involved in mammary fatty acid synthesis are Acetyl-coenzyme a (acetyl-CoA) carboxylase (ACC) and fatty acid synthase (FAS). ACC is a rate limiting enzyme and FAS is responsible for elongation of fatty acid chains.

1.3.6.1 Synthesis of CLA in ruminants

CLA in ruminant milk can originate from two potential sources: as intermediate products in the biohydrogenation of LA to stearic acid (C18:0) in the rumen; and from endogenous synthesis from vaccenic acid (C18:1 n-7) in the mammary glands. Vaccenic acid, the main ruminant *trans* fat[188], is an intermediate product in the biohydrogenation of LA and other C18 PUFAs (α- and γ-linolenic acid) to stearic acid in the rumen. The synthesis is catalysed by *delta*-9 desaturase (also known as stearoyl-CoA 9-desaturase)[189,190].

1.3.7 Promotion of *UCP1* expression by fatty acids

Fish oil, containing the omega-3 fatty acids docosahexaenoic acid (DHA, C22:6) and eicosapentaenoic acid (EPA, C20:5), has been found to induce *UCP1* expression in both BAT and WAT (presumably in brite adipocytes in the latter), increasing energy expenditure and reducing body weight and adipose tissue accumulation[191]. The mechanisms behind this increase in *UCP1* expression are thought to be due to the actions of fish oil on the sympathetic nervous system (SNS), where it increased urinary norepinephrine and norepinephrine turnover in BAT[191]. Norepinephrine binds with β_3 adrenergic receptors (the expression of which was also increased with fish oil intake[191]), which ultimately results in the activation of *UCP1* (Figure 1.2, Page 9). A similar study found that EPA/DHA supplementation was not effective in increasing UCP1[192]. However, these varying results could be partially explained by the lower concentrations of EPA used in the second study, as when human pre- and mature adipocytes are treated with EPA *in vitro*, *UCP1* expression is upregulated, an effect which was not seen with DHA[193].

Priego *et al.* found that dietary supplementation of rats in pregnancy and lactation with olive oil, which is high in MUFAs, decreased the rate of weight gain and increased UCP1 in the BAT of offspring at the time of weaning[194]. This effect could be attributed to milk oleic acid (C18:1 n-9) content, which demonstrated a positive correlation with UCP1. The effect of MUFAs on *UCP1* expression has also been seen in adult rats fed a high-fat diet[195] and, in humans, olive oil consumption results in the stimulation of diet-induced thermogenesis in obese post-menopausal women[196]. Diet-induced thermogenesis is the energy expended after consuming food. This is made up of the energy required to digest food, and the associated generation of heat, which expends some of the calories ingested[197,198].

1.4 Early life nutrition

1.4.1 Effects on adult health

1.4.1.1 The developmental origins of health and disease

The Developmental Origins of Health and Disease (DOHaD) concept emerged in the 1980s, from research published by David Barker and colleagues, in which epidemiological studies of birth and death records revealed a strong geographical relationship between infant mortality in 1921-25 and ischaemic heart disease, bronchitis, stomach cancer and rheumatic heart disease mortality rates in 1968-78[199]. A relationship between infant weight and later ischaemic heart disease mortality, implicated environmental influences which impair growth in early life as potential risk factors for ischaemic heart disease[200]. The authors later linked fetal undernutrition at different stages of gestation with low birth weight[201].

In 1962, Neel described the 'thrifty genotype' hypothesis[202], in which he proposed that people of a certain genotype were able to survive better in times of limited nutrient availability. This hypothesis would imply that, in modern times, where the majority of the Western world do not have the problem of limited nutrient availability, in fact, we generally have access to a more than adequate supply of food, the previously beneficial 'thrifty' genotype has negative effects, leading to NCDs.

In 1992, Hales and Barker proposed the 'thrifty phenotype' hypothesis[203], which suggested that sub-optimal pre- and postnatal nutrition could cause permanent alterations to certain tissues and organs, such as the pancreas, which could increase the later susceptibility to type 2 diabetes and the metabolic syndrome. They suggested that the adaptive response to poor nutrition in early life was to ensure survival in later life[204], assuming that nutrition would remain suboptimal in later life. Of course, whilst nutrition may be poor *in utero*, this does not mean that it will be so throughout a person's life. Therefore, when a person is exposed to malnutrition in early life, but is exposed to adequate, or excessive, nutrition in later life, their 'thrifty phenotype' becomes detrimental.

1.4.1.2 Nutritional programming

The concept of 'nutritional programming' is closely related to that of DOHaD. It was introduced by Lucas[205] who described the concept of diet or nutritional status in early life having a permanent or long-term effect on health[205].

A lot of research has been conducted into nutrition *in utero*, however, the focus of this thesis is early post-natal nutrition, specifically in the pre-weaning period, which can programme later obesity.

1.4.2 Breastfeeding

1.4.2.1 General health benefits

Human milk provides neonates with all the nutrients which they require, and whilst all of its properties are not fully understood, it is universally recognised as the best form of nutrition for human infants. Exclusive breastfeeding for the first 6 months of life is therefore recommended for all infants by the WHO[206]. Exclusive breastfeeding is defined by the WHO as an infant receiving "only breastmilk, and no other liquids or solids, with the exception of medicine, vitamins, or mineral supplements"[207]. Despite this, breastfeeding is not always possible, for a multitude of reasons. Whilst infant formula milk has improved over the last 60 years, studies continue to show that breastfed infants fare better than those who are formula fed. Being breastfed can potentially reduce the risk of many health complications, including: diarrhoea[208]; respiratory tract infection[209,210]; acute otitis media[211,212] ; high blood pressure[213,214]; high total cholesterol[215]; overweight and obesity[216,217]; type 1 diabetes[218]; type 2 diabetes[219]; necrotising enterocolitis[220,221]; childhood asthma[222-224]; and sudden infant death syndrome[225].

In addition to exclusive breastfeeding for the first 6 months of life, the WHO also recommend breastfeeding, alongside the introduction of appropriate complementary food until 2 years of age[206]. Breastfeeding beyond 6 months has been found to provide infants with additional protection against obesity[226].

With specific regard to fatty acids, infants who are breastfed have been shown to have higher proportions of certain lipids in their brains, retina and erythrocytes compared to formula fed infants, in particular, DHA and arachidonic acid (AA)[227]. This has been associated with the improved neurodevelopment which has been reported in breastfed, compared to formula fed, infants[228,229].

1.4.2.2 The variation of milk composition

Looking at the factors which affect the composition of human breast milk is challenging. The composition of an individual mother's breast milk varies over the course of each

feed[230], each day[231], and over the course of lactation[230,232-234]. In addition, there are substantial differences in breast milk composition between women.

Fat is the main source of energy in human breast milk and appears to be the macronutrient in breast milk which is most variable between women[235,236]. As in ruminants, the fatty acid composition of human breast milk is affected by the mother's diet. A study by Peng *et al*. analysed fatty acid intake from the diets of women in the third trimester of pregnancy and collected breast milk samples 5 days after they gave birth, finding that the fatty acid content of breast milk was reflective of that in the mother's diet[237]. Other studies, in humans [238-240] and animals [241,242], have also found that dietary factors can influence the fatty acid composition of milk which, in turn, can impact upon infant growth and development[243-245].

1.4.2.3 Protective effects against obesity

It seems that in very early life there is a critical window for the development of obesity later in life[246]. Human milk and cow's milk (which is modified and used for many infant formula milks) are known to have different compositions[179], with cow's milk containing higher protein to support the rapid growth of the offspring[247]. In contrast, early growth in humans is generally much slower, with substantial increases in brain volume[248], and human milk contains a higher proportion of fat[249]. It is possible that variations in milk fat could be a factor affecting adipose tissue growth and development, which could programme later obesity risk[250].

A growing body of research has found breastfeeding to have a protective effect against overweight and obesity[251-257], however, other studies have not made this association[258,259]. A meta-analysis by Yan *et al.,* which analysed the results of twenty-five studies, with over 200,000 participants, concluded that breastfeeding is associated with a 22% reduction in the risk of childhood obesity[260]. It seems likely that the type of milk received in early life is a factor which contributes to later risk of obesity.

Several mechanisms for this apparent protective effect have been proposed. Protein intake is lower in breastfed than in formula fed infants[261], and increased protein intake in infancy has been associated with obesity in later life[262]. Although those who were breastfed consume a healthier diet in childhood than those who were formula fed[263,264], adjustment for diet does not appear to change the magnitude of the relationship between breastfeeding and obesity[263]. Additionally, a study by Victora *et al.* suggests that the

consumption of a healthier diet in breastfed individuals may not extend to adulthood[265], therefore, this mechanism is unlikely to be the main cause of protective effects.

Bioactive factors in breast milk are likely to play a role in the protection breastfeeding offers against obesity. Leptin (Section 1.2.2, Page 5) is present in human breast milk[266], yet its presence in infant formula milk is variable and not well researched[267,268]. Differences have been found in the plasma leptin concentrations of breast and formula fed infants[269], which may support the hypothesis that differences in body weight of breast and formula fed infants are partially due to different endocrine responses to bioactive substances present in breast milk which are critical in metabolic development, however, could also simply reflect the response to different nutrients present in breast/formula milk. A review by Symonds *et al.* suggested that the composition of milk may play a role in BAT function[111], suggesting that factors present in breast milk (such as prolactin[270]), but absent from infant formula milk, could cause differences between energy regulation in breast and formula fed infants. Prolactin has been found to have a thermogenic effect in large animal studies[94], and therefore could be involved in the protective effects on obesity.

Behavioural factors may also contribute to the protective effect of breastfeeding on obesity, with infants who were bottle fed (infant formula milk or expressed breast milk) in early infancy being more likely to empty the bottle (or cup) later in infancy than those who were breastfed[271]. This suggests that due to the nature of breastfeeding (feeding on demand, and parents not being as aware of the quantity of milk consumed with each feed), breastfed infants develop a better ability to self-regulate their milk intake.

It is likely that breastfeeding protects against obesity by more than one of these mechanisms. Whilst it is clear that obesity is caused by a multitude of factors, the high rates of formula feeding, especially in the UK, alongside the numerous other positive effects associated with breastfeeding, suggest that research into the differences between infant formula and human breast milk, and thus ways to bridge this gap, is an area worth pursuing.

1.4.2.4 Statistics on breastfeeding

The prevalence of breastfeeding is defined as the percentage of babies who are being breastfed at specific ages. Breastfeeding, in this case, can be in addition to feeding by infant formula milk and/or solid food.

In the UK in 2010, breastfeeding was initiated (that is, the baby was put to the breast at least once) in 81% of babies on average, however, rates of initiation vary widely, being as low as 36.6% in some areas of the country[272]. By one week of age, the average prevalence of breastfeeding drops to 69%, then again to 55% at six weeks, and by six months the prevalence is around 34%.

Exclusive breastfeeding is defined by the WHO as an infant receiving "only breastmilk, and no other liquids or solids, with the exception of medicines, vitamins, or mineral supplements"[207]. In 2010, at birth, 69% of babies in the UK were exclusively breastfed. These rates drop rapidly, with only 46% of babies still being exclusively breastfed by 1 week of age, 23% at six weeks, 17% at three months and just 12% are still being exclusively breastfed at four months of age[273].

The higher prevalence of breastfeeding, compared with exclusive breastfeeding, highlights the popularity of 'mixed-feeding' (giving both breast and infant formula milk), with rates of mixed feeding increasing in the UK between 2005 and 2010[273,274]. The use of infant formula milk amongst mothers who either 'mix-feed' or exclusively formula feed is high in the UK, with 9 out of 10 mothers having given their baby milk other than breast milk by the age of six months[273].

Breastfeeding rates appear to be influenced by several factors, including: age of mother, with younger mothers being less likely to breastfeed; socio-economic class (SEC) of mother, with mothers from higher SECs having higher breastfeeding rates; education of mother, with mothers who completed full-time education at younger ages being less likely to breastfeed; and geographical location, with mothers from the most deprived areas being less likely to breastfeed[273].

Conversely, worldwide there seems to be an opposite trend, with breastfeeding prevalence at 6 months showing a strong inverse correlation with income (measured in terms of gross domestic product per person)[275]. The prevalence in most high-income countries is below 20%, whereas the prevalence is much higher in sub-Saharan Africa, south Asia and areas of Latin America[275]. Figure 1.8 shows the global distribution of breastfeeding at 12 months of age.

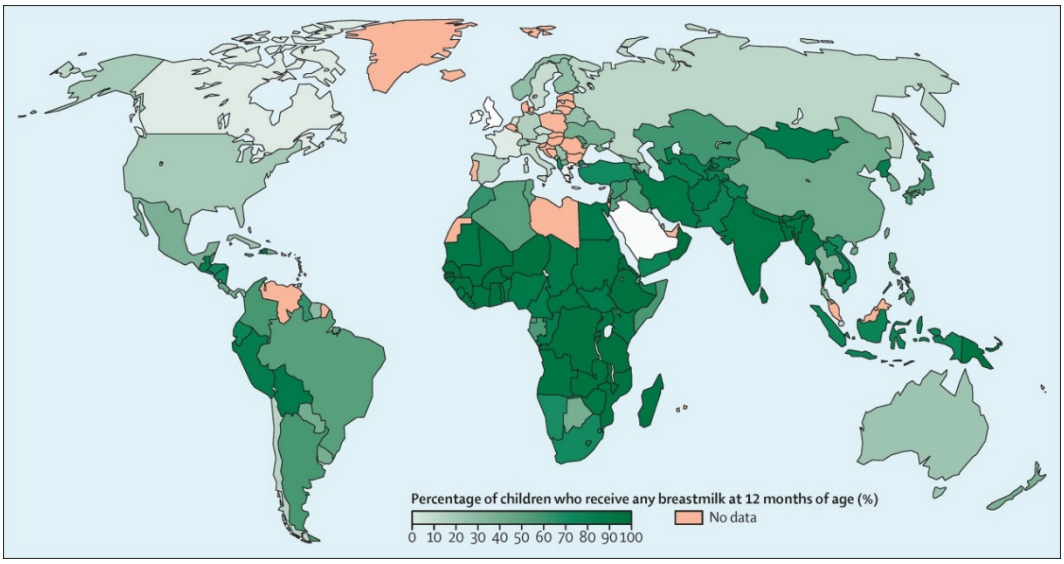

Figure 1.8 The global distribution of breastfeeding at 12 months of age.

Data from Victora *et al.*[275] collected from 153 countries between 1995 and 2013.

1.4.3 Formula feeding

1.4.3.1 Production of formula milk

The key aspect of infant formula milk production in relation to my investigation is the fat content. Generally, it can be difficult to obtain specific details on the production and specific fat profile, due to the competitive nature of the industry.

The fat used in infant formula milk, often referred to as the 'fat blend', comes from two main sources: vegetable oils, which are adjusted and blended to more closely resemble the basic fatty acid composition of human milk fat (HMF); or a mixture of vegetable oil and milk fat[276,277]. Despite the milk fat from non-human mammals having more similarities to human breast milk than vegetable oil[276], the use of a vegetable oil fat blend in infant formula is more common[276]. Although, recently there has been a push for more infant formula milk to contain ruminant milk fat[278], not least because the positioning of fatty acids within a triglyceride (detailed in 1.3.4) in bovine milk is more similar to HMF than in vegetable fat[171,174,175]. The fat globules of HMF are spherical with a three-layer membrane, and a similar structure is also similar in the milk of other mammals such as cows[277]. Research indicates that making the structure of fat in infant formula milk more similar to HMF could have beneficial effects on the infant, including their digestion and gut microbiota[277]. Therefore, it has been proposed that the addition of cow's milk fat would be beneficial in

this context[277], suggesting that it may be better to take milk fat, and modify it appropriately to be more similar to HMF, rather than use a complete vegetable oil blend[276].

1.4.3.2 Reasons for formula feeding

There are many personal reasons why a woman may choose not to breastfeed, or to mix feed[273]. However, these aside, there are other reasons why a baby may be given infant formula milk. There are some medical conditions which may prevent or discourage mothers from breastfeeding in developed countries, for example, HIV. There are also some medications which, if taken by the mother, can transfer to the breast milk, making it unsafe for the baby. In addition to medical reasons, sometimes practical reasons can prevent a baby being breastfed, for example, if the baby is adopted. In these cases, infant formula milk is the best alternative. It is important to understand that, whilst in a perfect world every baby would be breastfed exclusively for the first 6 months of life, in reality, this is not the case. This makes it vital that the alternative, infant formula milk, is the best possible substitute. Therefore, alongside the continuous encouragement to increase breastfeeding rates, it is imperative to carry out careful research into exactly which components of human breast milk exert its beneficial effects. Once these components are identified, they can be incorporated into infant formula milk in order to help improve the long-term health outcomes of formula fed infants, making them more comparable to those of breastfed infants.

1.4.4 The role of dietary fat in early life on programming of obesity

The majority of studies in early life nutrition have focussed on under nutrition, protein restriction and/or over nutrition[279]. Most studies relating to fat have been confined to the effects of high or low-fat diets[280], and have not focused on the quality of fat. However, fat is more than simply energy, with different fatty acids performing different biological functions.

An interesting study by Widdowson *et al.* in 1975[281] compared adipose tissue fatty acids between British and Dutch infants in early life. At this time, infant formula milk in the UK was mainly based on unmodified cow's milk and contained approximately 2% LA, whilst in the Netherlands it was prepared with vegetable oil and contained around 60% LA. This was reflected in the LA content of the infant's adipose tissue, which was far higher in the Dutch formula fed infants at 4 months (32-37% of fat) compared to the British infants, where LA adipose tissue content never exceeded 3% of fat. This is despite the adipose tissue content

of LA in both British and Dutch infants both being 1-3% of fat at birth. This was the first real step in realising that the quality of fat consumed had a direct effect on that which was incorporated into an infant's adipose tissue.

As discussed previously, adipogenesis is the process by which cells differentiate and preadipocytes become adipocytes (Section 1.2.8.1, Page 12). The omega-6 AA appears to be very adipogenic, and was identified as the main adipogenic component of fetal bovine serum (FBS)[282]. Brief exposure of preadipocytes to the LCFAs in FBS is sufficient to trigger proliferation of preadipocytes and the expression of genes related to differentiation[283]. Further *in vitro* work has shown that AA increases production of cAMP and triggers terminal differentiation of adipocytes[282]. Furthermore, AA synthesises prostaglandins, which have also been shown to modulate the differentiation of preadipocytes *in vitro*[284,285], and which activate PPARs. In turn, *PPARγ* agonists have been found to enhance the potency of the AA mediated pathway in promoting adipogenesis[286]. *In vivo*, mice fed a diet high in LA during gestation and lactation had offspring with higher body weight and fat mass than those of mothers fed a diet with a high LA:LNA ratio, and the difference in body weight was maintained until adulthood[287]. In contrast to the omega-6 LA, the omega-3 LNA plays an anti-adipogenic role, by altering the production of AA by competitive inhibition of Δ^6 desaturase, for which LA and LNA are both substrates. A high dietary LNA:LA ratio seemingly downregulates the conversion of LA to AA[287]. *In vivo*, LA and its derivative AA have both been associated with increased adiposity in young animals[286,288,289].

In the early life diet of human infants, the type of milk received affects the intake of LA, which constitutes approximately 18% of fat in infant formula milk, higher than the average 12.7% LA content found in the breast milk of women in Europe[250]. Interestingly, the LA content of breast milk has increased in the USA from 6-7% of fat in 1944, to around 10% in the 1960s and 15% in 1990[290]. Similarly, in the UK, breast milk contained around 7% LA in the 1960s, which increased to 11-12% in the 1990s[290]. The amount of LNA in breast milk has remained much more constant[290], leading to an increased LA:LNA ratio in breast milk. The fatty acids present in breast milk largely reflect those in the mother's diet[291], in both the short- and long-term, with a study finding that 70% of LA in breast milk comes from maternal body stores (long-term) and 30% directly from the diet (short term)[292]. Ailhaud *et al*. suggest that dietary omega-6 PUFAs (of which LA is most abundant) in early life could contribute to obesity, due to their observed increases in dietary PUFAs (likely to be omega-6) in infants in the USA compared with France[290]. They commented that there were no clear

differences in energy intake between infants in Europe and the USA, yet rates of childhood obesity were higher in the USA.

1.5 Conjugated linoleic acid

1.5.1 Introduction

Conjugated linoleic acid (CLA) is a collective term for a group of positional and geometric isomers of LA, first positively identified by Ha *et al.* in 1987[293].

The term 'conjugated' refers to the arrangement of the double bonds in isomers of LA (CLA). Conjugated bonds refer to double bonds separated by a single bond. The conversion of LA to CLA which occurs when a chemical or microbial reaction causes one or both of the double bonds of LA (C18:2 *cis*-9, *cis*-12), which are not conjugated (as they are separated by two single bonds), to move and/or change configuration, forming a conjugated isomer, the two biologically active isomers' structure can be seen in Figure 1.9, and they can have different, even opposing, effects on health.

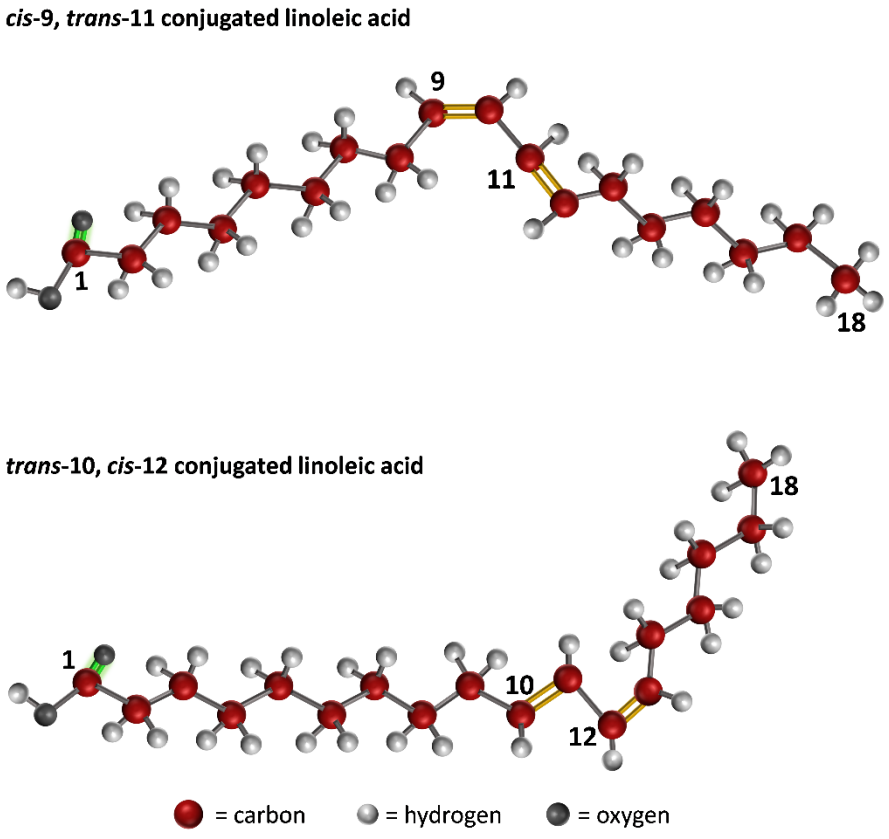

Figure 1.9 the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated linoleic acid isomers.

The diagram shows their 18 carbon chains with double bonds present at carbons 9 and 11 in the *cis*-9, *trans*-11 isomer and carbons 10 and 12 in the *trans*-10, *cis*-12 isomer.

1.5.2 Effects of CLA on health

According to common nomenclature CLA is a TFA, because most of its constituent isomers will have at least one double bond in the *trans* configuration. However, as discussed in Section 1.3.2.1 (Page 17), it is a ruminant TFA and these have not been associated with the negative health effects which are found with industrial TFAs[151-157]. In fact, CLA first became of interest for its health benefits as an anticarcinogen[294-296]. Subsequent research in animals has identified numerous beneficial biological responses to CLA, including reduced fat deposition and increased lean body weight[297-299], improved immune function[300,301], and reduced atherosclerosis[302,303]. CLA has also attracted interest for its role in the management of type 1[304] and type 2[305,306] diabetes, due to its ability to increase adipocyte insulin sensitivity[307].

Much of the research into CLA has been carried out using a mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12, however, some research has suggested that they exert opposing effects. For example, one study found that the *trans*-10, *cis*-12 isomer increased, and the *cis*-9, *trans*-11 isomer decreased, LDL:HDL plasma cholesterol[308].

Many of the commercially available CLA supplements contain approximately equal quantities of the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 isomers. However, as previously mentioned, the *cis*-9, *trans*-11 is by far the most abundant naturally occurring isomer in the human diet.

Larger quantities of the *trans*-10, *cis*-12 isomer have been associated with some negative side effects in mice, including hyperinsulinemia[309], insulin resistance[309,310] and liver steatosis[309]. As in mice, in abdominally obese men, larger doses of the *trans*-10, *cis*-12 isomer have also been found to induce insulin resistance[311]. A study in postmenopausal women found that a CLA mixture containing approximately equal quantities of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers induced negative health effects, increasing markers of coronary vascular disease[312]. These results were not seen in a supplement rich in the *cis*-9, *trans*-11 isomer[312]. Furthermore, a study in mice which found that the *trans*-10, *cis*-12 isomer increased insulin resistance also found that the *cis*-9, *trans*-11 isomer prevented it from occuring[310]. Again, from an evolutionary perspective, the lack of negative health effects associated with the *cis*-9, *trans*-11 isomer, are not particularly surprising, given that it is naturally present in human breast milk.

The majority of CLA isomers (those with a double bond in the *trans* configuration) are TFAs, and to be more specific, ruminant derived TFAs, which are explained in Section 1.3.2 (page 16).

1.5.3 Effects of CLA on body composition

Studies supplementing rodents with a mixed CLA supplement (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 in approximately equal quantities) have found it to reduce body fat and increase lean mass[307,313-318]. Azain *et al.* observed that the decrease in fat mass was due to a decrease in adipocyte size, rather than number[318]. Some studies have attributed this fat reduction to associated increased energy expenditure in BAT[314], potentially caused by the increases which have been found in *UCP1* expression with mixed CLA supplements (Table 1.2, Page 36). However, others have not found increased *UCP1* expression to be a determining factor[317,319].

In humans, mixed CLA supplements have also been shown to decrease body fat in obese and overweight[320], as well as normal weight[321,322], men and women. However, other studies have not found CLA to affect body fat[323] or BMI[324,325]. Differences in results between studies supplementing CLA are likely, at least in part, to be due to variations in CLA doses, the duration of supplementation, and the ratios of individual isomers.

Studies have been conducted in an attempt to distinguish which isomer is responsible for any effects on adiposity. Gavino *et al*. supplemented hamsters on a high SFA diet with *cis*-9, *trans*-11 CLA, mixed isomer CLA, or LA, and found that the mixed supplement caused significantly lower rates of weight gain than the *cis*-9, *trans*-11 diet, despite increased food intake[326]. This suggests that the *cis*-9, *trans*-11 isomer is not responsible for the decrease in body fat found with mixed CLA supplements. A study which supplemented mice with the *trans*-10, *cis*-12 isomer found that it decreased body fat to a greater extent than a mixed supplement[327]. Martins *et al.* treated adipocytes with *cis*-9, *trans*-11 and *trans*-10, *cis*-12, both individually and as a mixture, and reported that lipogenesis and differentiation, along with adipocyte triglyceride content, seemed to be reduced by the *trans*-10, *cis*-12 isomer[328]. This would make sense in evolutionary terms, as the *cis*-9, *trans*-11 isomer is the most abundant in breast milk and, by its very nature, breast milk's primary purpose is to enable an infant to grow, so for a factor present within it to induce weight loss would be disadvantageous. Nevertheless, the effect of individual isomers on body fat in humans has not been well studied.

1.5.4 Effect of CLA on *UCP1* expression

There has been some promising research suggesting that CLA could be involved with BAT function. Table 1.2 summarises studies which have investigated the effect of mixed and individual CLA isomer supplementation on *UCP1* expression. Whilst results are variable, overall they suggest that CLA does have an effect, with several studies finding that various doses of CLA supplements increase *UCP1* mRNA expression[97,329-334]. However, some studies have found no effect[317,319], and others have found that CLA decreases *UCP1* expression[316,335].

The inconclusive results regarding the effects of CLA on *UCP1* expression are likely to be partially due to the use of mixed isomer supplements. Although, the few studies which have investigated the isomers' effects in isolation have also yielded mixed results, with four studies finding increased *UCP1* mRNA with *trans*-10, *cis*-12 supplementation[97,330,331,334]. However, a study by Rodríguez *et al.* found that the *trans*-10, *cis*-12 isomer inhibits *UCP1* expression[336], and this is backed up by Metges *et al.,* who found a 75% decrease in *UCP1* mRNA expression in primary brown adipocytes with *trans*-10, *cis*-12 treatment[332]. Interestingly, Rodríguez *et al.* and Metges *et al.* also investigated the effects of the *cis*-9, *trans*-11 isomer, and both found that this isomer increased *UCP1* expression[332,336]. Shen *et al.* supplemented mice with the *trans*-10, *cis*-12 isomer, either alone, or in combination with the *cis*-9, *trans*-11 isomer, and found that both supplements increased the mRNA expression of *UCP1* in WAT, and that the *cis*-9, *trans*-11 isomer had no additional effect[334]. This study also investigated the effects of the CLA dose size on UCP1, and found that higher doses did not enhance UCP1[334]. A weakness of this study, with respect to investigating the individual isomers, is that whilst the dose of the *trans*-10, *cis*-12 isomer remained constant, the *cis*-9, *trans*-11 isomer dose, and therefore the ratios of the two isomers varied between the different treatments.

Ealey *et al.* found a species-specific effect of a mixed isomer CLA supplement, which induced an increase in UCP1 in rats, but a decrease in mice, for which the causes were unclear[337]. The effects of CLA specific to BAT, to my knowledge, have not been investigated in larger mammals or humans.

Another limitation of current research is that the majority of studies used mRNA expression to determine the effect of CLA on *UCP1*. In fact, only 4 of the studies in Table 1.2 measured UCP1 protein[331,332,334,337], and these yielded more consistent results. The previously mentioned Ealey *et al.* study found an increase in BAT UCP1 in rats and Wendel *et al.* found that a mixed supplement increased UCP1 protein in WAT[333]. Zhai *et al*. looked at the effects

of the isomers individually in cells and found both the *cis*-9, *trans*-11 and (to a greater extent) the *trans*-10, *cis*-12 isomer increased UCP1 protein[331]. Shen *et al*. also found that UCP1 protein was increased in WAT with both a mixed supplement and the *trans*-10, *cis*-12 isomer[334]. The relationship between mRNA expression and protein is not as clear-cut as is often assumed. Exclusive use of mRNA to reflect functional protein abundance has been criticised[338], as there are numerous factors between the transcription of mRNA and translation to the protein which can cause results of the two measures to differ[339]. With this in mind, more studies measuring UCP1 protein would be beneficial.

A clear mechanism for the effects of CLA on *UCP1* has not been identified. Wendel *et al.* report that the increase in UCP1 was not through the actions of *ADRB3*[333], and the study by Rodríguez *et al.* found the increase in UCP1 with *cis*-9, *trans*-11 to be induced by norepinephrine[336].

Study	Model	Conjugated linoleic acid isomer(s)	Dose of supplement (% of diet w/w)	Duration	Effect on *UCP1*
Ealey *et al.* (2002)[337]	Rats and mice 5 weeks (of age) Female	Mixed (43% *cis*-9, *trans*-11 /*trans*-9, *cis*-11 and 45% *trans*-10, *cis*-12)	1%	3 weeks	Mice : ~40% ↓ mRNA and ~40% ↓ protein in BAT Rats: ↔ mRNA and ↑ protein in BAT.
Takahashi *et al.* (2002)[335]	Mice 5 weeks Male	Mixed (32.9% *cis*-9, *trans*-11 and 33.7% *trans*-10, *cis*-12)	2%	3 weeks	36% ↓ mRNA in BAT.
Tsuboyama-Kasaoka *et al.* (2000)[316]	Mice 7 weeks Female	Mixed (34% *cis*-9, *trans*-11 and 36% *trans*-10, *cis*-12)	1%	4 days to 8 months	↓ mRNA in BAT.
West *et al.* (2000)[319]	Mice 5 weeks Male	Mixed (39% *cis*-9, *trans*-11/*trans*-9, *cis*-11 and 41% *trans*-10, *cis*-12)	1%	5 weeks	↔ mRNA in BAT or WAT.
DeLany *et al.* (2000)[317]	Mice 4 weeks Male	Mixed (39% *cis*-9, *trans*-11/*trans*-9, *cis*-11 and 41% *trans*-10, *cis*-12)	1%	5 weeks	↔ mRNA in BAT.
Peters *et al.* (2001)[329]	Mice 10-12 weeks Male	Mixed (42% *cis*-9, *trans*-11/*trans*-9, *cis*-11 and 44% *trans*-10, *cis*-12)	0.5%	4 weeks	10-fold ↑ mRNA in WAT.
Continued overleaf					

House *et al.* (2005)[330]	Mice 9 weeks Male	*trans*-10, *cis*-12	1%	2 weeks	12-fold ↑ mRNA in BAT.
LaRosa *et al.* (2006)[97]	Mice 9 weeks Male	*trans*-10, *cis*-12	0.5%	2 weeks	26-fold ↑ mRNA in WAT.
Zhai *et al.* (2010)[331]	3T3-L1 cells	*cis*-9, *trans*-11 or *trans*-10, *cis*-12	NA	NA	*cis*-9, *trans*-11 : 100%↑ mRNA and ↑ protein (% not specified). *trans*-10, *cis*-12: 400% ↑ mRNA and 150% ↑ protein.
Metges *et al.* (2003)[332]	Primary brown adipocytes	*cis*-9, *trans*-11 or *trans*-10, *cis*-12	NA	NA	*cis*-9, *trans*-11: 1.68-fold ↑ mRNA. *trans*-10, *cis*-12: 75% ↓ mRNA.
Wendel *et al.* (2009)[333]	ob/ob mice 6 weeks Female	Mixed (39% *cis*-9, *trans*-11 and 39% *trans*-10, *cis*-12)	1.5%	4 weeks	↑ mRNA and ↑ protein in WAT.
Shen *et al.* (2013)[334]	Mice 5-6 weeks Male	Mixed or *trans*-10, *cis*-12	0.06, 0.2 or 0.6%	3 weeks	Both supplements ↑ mRNA and ↑ protein in WAT, but not in the higher dosage. Both supplements ↔ mRNA and ↔ protein in BAT.
Rodríguez *et al.* (2002)[336]	Primary brown adipocytes	*cis*-9, *trans*-11 or *trans*-10, *cis*-12	NA	NA	*cis*-9, *trans*-11: ↑ *UCP1* induction by norepinephrine. *trans*-10, *cis*-12: ↓ *UCP1* induction by norepinephrine.

Table 1.2 Summary of studies investigating the effect of conjugated linoleic acid (CLA) on uncoupling protein 1 (UCP1).

Upward arrows (↑) indicate that a significant increase was found, downward arrows (↓) indicate that a significant decrease was found and ↔ indicates that no significant changes were observed.

1.5.5 Dietary sources of CLA in humans

The synthesis of CLA in ruminants means that the CLA in the human diet comes almost exclusively from ruminant meat and milk products [340-342]. Ritzenthaler *et al.* reported that the majority of CLA in the human diet comes from dairy products, followed by beef[343] (Figure 1.10). They also found that the majority of dietary CLA is in the form of the *cis*-9, *trans*-11 isomer (around 80-90% of total CLA[343]).

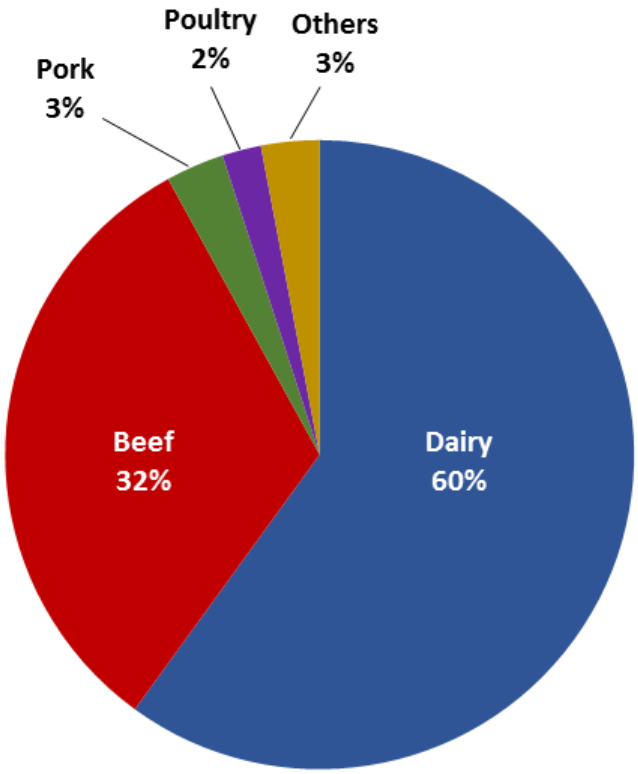

Figure 1.10 Percentages of conjugated linoleic acid in the human diet that come from each dietary source.

Data from Ritzenthaler *et al.*[343], collected from participants in the USA.

Human CLA intake is likely to have declined in recent decades, with studies showing a decline in the consumption of dairy products[344-348]. In addition to this, a trend has emerged for substituting full-fat dairy products and meats with lower fat versions[344-347,349] in the USA and Europe, which will inevitably reduce CLA intake, a component of the fat. It has been postulated that there is an inverse relationship between the consumption of dairy foods and obesity. Studies investigating the relationship between dairy consumption and body weight have yielded inconclusive results. Several studies have reported an inverse association between dairy consumption and body weight[350-352] whilst other studies have reported no significant relationship between body weight and dairy consumption [353-356]. However, in correlational studies like this, it is very difficult to account for potential confounders.

1.5.6 Seasonal variability of CLA in milk

Several studies show a seasonal effect on milk composition. This effect is thought to be, at least in part, attributable to dietary changes driven by season. The winter diet generally contains no fresh grass and has more concentrate and grass silage than the summer diet[184,357]. Grass used for silage undergoes wilting and ensiling, which causes extensive lipolysis and oxidative loss of fatty acids[358]. In addition to this, the grass used for silage is generally more mature than that consumed by grazing animals, which leads to further decreases in PUFA levels[359]. These changes in diet cause milk from cows grazing in the summer to be lower in SFAs and higher in CLA than those fed a silage-based diet in winter[360]. In fact, CLA appears to be the aspect of milk fat most susceptible to seasonal variation, with numerous studies showing that milk CLA concentrations increase in the summer months (Table 1.3). This seasonal effect will almost certainly mean that human dietary CLA intake is subject to seasonal variation. The numerous factors affecting the CLA content of milk also mean that in the infant formula milks which include dairy fat, CLA content is likely to be variable.

Study	Species	Total fat	MUFAs	PUFAs	Total SFAs	Medium-chain SFAs	Long-chain SFAs			CLA			*TFAs*	Omega-3 FAs	Omega-6 FAs
							C14:0	C16:0	C18:0	Total CLA	*c*-9, *t*-11	*t*-10, *c*-12			
Lock *et al.*2003 [361]	Cows	↑	↑	↑	↓	↓	↔	↓	↓	↑	↑	↔	/	/	/
Heck *et al*.2009 [360]	Cows	↓	/	/	↓	↓	↓	↓	↑	↑	↑	/	/	/	/
Collomb *et al*.2008 [362]	Cow	/	↑	↑	↓	↓	↓	↑	↓	↑	↑	↔	↑	↔	↔
Talpur *et al*.2008 [363]	Cow	/	↑	↔	↓	↓	↓	↑	↓	↑	↑	↑	↑	↑	↔
Talpur *et al.*2008 [363]	Buffalo	/	↑	↑	↓	↓	↓	↑	↓	↑	↑	↑	↑	↔	↑
Talpur *et al*.2008 [363]	Goat	/	↑	↑	↓	↓	↓	↑	↓	↑	↑	↑	↑	↑	↑
Talpur *et al*.2008 [363]	Sheep	/	↑	↑	↓	↓	↓	↑	↓	↑	↑	↔	↑	↔	↑
Fernandez-Garcia *et al*.2006 [364]	Sheep	/	/	/	/	↓	↓	↓	↑	/	/	/	/	/	/
Melunchova *et al.* [365]	Sheep	/	/	↑	↓	↓	↑	↑	↔	/	↑	/	/	↑	↓

↑ Increase in summer months ↓ Decrease in summer months ↔ No change / Not measured

Table 1.3 Summary of studies investigating the effect of season on the fatty acid profile of ruminant milk.

MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, SFA = saturated fatty acid, CLA = conjugated linoleic acid, TFA = *trans* fatty acid, FA = fatty acid.

1.5.7 CLA content of milk in early life

1.5.7.1 Human breast milk

Studies investigating the CLA content of human breast milk are summarised in Table 1.4.

Most reported that the *cis*-9, *trans*-11 isomer makes up 80-100%[366-368] of total CLA, however, one study in Spain by Luna *et al*. found much lower percentages of *cis*-9, *trans*-11 (>60%)[369]. This study only used milk samples from 4 women, whereas other studies, which found a higher percentage of *cis*-9, *trans*-11, such as Mosley *et al.* who tested 81 samples and found that *cis*-9, *trans*-11 made up ~80% of CLA[366] had larger numbers. Few studies have been conducted into the amount of the *trans*-10, *cis*-12 isomer in breast milk, and the only study which reported absolute values for this isomer was by Luna *et al*., who reported that *trans*-10, *cis*-12 contributed only 1.5% of total CLA[369]. Others suggest that the concentration of the *trans*-10, *cis*-12 isomer in breast milk is low, as only the *cis*-9, *trans*-11 isomer was detected[367,368], indicating that if other isomers were present, they must have fallen below detection limits.

Mothers consuming a diet containing organic meat and dairy products have higher levels of *cis*-9, *trans*-11 CLA present in their breast milk[370]. Several other studies have looked at relationships between maternal dairy fat consumption and the amount of CLA found in breast milk and there is a dose-response relationship between a women's dietary CLA and the CLA content of her milk [371-375].

As shown in Table 1.4, the amount of CLA present in breast milk is subject to large individual variation between women. Maternal diet is a large factor in determining milk CLA content, however, it is estimated that around 10-20% of the CLA in breast milk is endogenously synthesised by the desaturation of vaccenic acid to *cis*-9, *trans*-11 CLA in the mammary gland[376,377]. This suggests that the mammary gland may have evolved to synthesise CLA to perform some type of useful function for the mother or baby.

Study	Country	Subjects	Total CLA (% of fat)	*Cis*-9, *trans*-11 (% of fat)	*Cis*-9, *trans*-11 (% of total CLA)
Mosley *et al.* (2005)[366]	USA	n=81	0.27 - 0.79%	0.20-0.66%	Not reported
Oleynik *et al.* (2013)[378]	USA	n=7	Not reported	0.12-0.35%	Not reported
Jensen *et al.* (1998)[367]	USA	n=5	0.14-0.28%	0.14-0.28%	100%
McGuire *et al.* (1997)[368]	USA	n=14	0.22-0.54%	0.22-0.54%	83-100%
Luna *et al.* (2007)[369]	Spain	n=4	0.12-0.15%	0.08-0.14%	>60%
Precht *et al.* (1999)[379]	Germany	n=40	Not reported	0.23-0.63%	Not reported
Rist *et al.* (2007)[370]	The Netherlands	n=312	0.31-0.41%	0.25-0.34%	Not reported

Table 1.4 Summary of studies investigating the conjugated linoleic acid (CLA) content of human breast milk.

Whilst work has been carried out in order to determine the natural CLA content of human breast milk, very few intervention studies have been carried out in which lactating human mothers are given artificial CLA supplementation. Mosley *et al.* carried out a double-blind, 3x3 Latin square design trial, where 12 lactating women were given 2g/day of safflower oil, 2g/day of CLA plus 2g/day safflower oil, and 4g/day of CLA[372]. The CLA supplement used was made from equal quantities of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers. A dose-dependent increase in *cis*-9, *trans*-11 milk fat concentration with CLA supplementation was observed. Interestingly, the macronutrient composition of milk did not change with mixed isomer CLA supplementation, which is contrary to the findings of animal studies[380-384], although the sample size in the Mosley *et al.* study may have been too small to detect any differences (n=12). This result was also contrary to the only other CLA supplementation trial on lactating women to date, where Masters *et al.*[374] performed a double-blind placebo controlled cross-over study in 9 lactating women who received either 1.5g of a mixed CLA supplement containing mainly *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA or a placebo. The study showed that milk fat content was significantly lower in the CLA treatment period and recommended that lactating women should not take commercial CLA supplements. Whilst supplementation of CLA in adults is generally recognised as safe[385,386], it appears that this

may be different in lactating women. The possible risks associated with manipulating the CLA composition of breast milk, which may be a human infant's sole source of nutrition, could explain why the number of trials of CLA supplements in lactating women is low. With CLA supplementation inducing changes in the macronutrient composition of milk[374,380-384,387-390], which are likely to be unfavourable to the infant, it would be unwise to give these supplements to breastfeeding women.

1.5.7.2 Infant formula milk

There is a paucity of literature available on the amount of CLA present in infant formula milk. To date, I have only found three studies which report such data.

The first, conducted in 1997 by McGuire *et al*., measured the amount of total CLA and the *cis*-9, *trans*-11 isomer in 16 samples of infant formula milk (from four brands)[368]. Results showed that CLA was detectable in 37.5% of the samples tested, and concentrations ranged from undetectable to 0.2% of fat. Of those samples in which CLA was detected, the amount of CLA made up from the *cis*-9, *trans*-11 isomer ranged from 80% to 100% of total CLA. The same study also measured total CLA in human breast milk (0.22-0.54% of fat) which surpassed those found in the infant formula milk. However, the results from this historical study are not likely to be indicative of the amount of CLA that may be present in infant formula milk today.

Chin *et al.*[341] tested a variety of food for CLA content, and amongst these were 6 samples of infant formula milk. Unfortunately, the only information given on the samples was that 3 were from formula milk containing soy protein, and three contained milk protein. No details of fat sources were given, but the results suggest that milk fat was not the source, as in contrast to the findings of McGuire *et al,* of the low levels of CLA detected (0.03-0.07%) of fat*,* the *cis*-9, *trans*-11 isomer was not detected in any samples. Again, this is a relatively old study, conducted in 1992, so the relevance of its findings to modern day infant formula milk is questionable. However, it does suggest that there is a difference in the proportions of CLA isomers in human breast milk and infant formula milk.

A more recent study, published in 2013 in the USA[378], measured the total CLA content of four types of infant formula milk (Enfamil LIPIL®, Nestle Good Start Gentle Plus®, Similac Advance Early shield®, and SimilacIsomil advance® soy). CLA was undetectable in all formula milk tested. Unfortunately, the authors did not include the lower detection limits of their analyses. The main weakness of this investigation is the small number of infant

formula milks tested, which means that results are unlikely to be representative of infant formula milk as a whole. In addition, all of the infant formula milks tested were from the USA, and therefore not necessarily applicable to infant formula milk worldwide.

With the aim of improving the similarities between human breast milk and infant formula milk, more research in this area is important, to establish CLA concentrations in infant formula milk.

1.5.7.3 Legislation surrounding CLA specific to infant formula milk

Whilst, as previously mentioned (Section 1.3.2.1, Page 17) the EFSA impose no limits on TFAs in general food, the Commission Directive 96/4/EC states that TFAs must not exceed 3% in infant formula and follow on milk[391]. This Directive makes no distinction between ruminant and industrial TFAs. The natural levels of CLA in breast milk fall well within the 3% TFA limit imposed on infant formula milks, with measures reportedly ranging between 0.27% and 0.79% of total fatty acids, thus CLA could be legally included in infant formula milk in these quantities. However, at this time, it is not permitted for CLA to be added to infant formula milk in addition to that which is already present in the fat ingredients. The pertinent issue is that there is no distinction between industrial and ruminant derived TFAs in the legislation. This means that two formulas could both contain the same levels of TFAs, but these could be made up of very different proportions of ruminant and industrial TFAs, which potentially could produce different, even opposing, health effects. With regard to CLA, vegetable fat, which is commonly used in infant formula milk[276], the percentage of *cis*-9, *trans*-11 accounts for only around 40% and the *trans*-10, *cis*-12 isomer also makes up around 40% [341], which is very different CLA isomer ratios to human and ruminant milk fat (where *cis*-9, *trans*-11 accounts for 80-90% of total CLA). For this reason, when comparing the differences between CLA in breast milk and infant formula milk, the ratio of the isomers, as well as the total amount of CLA, must be considered. More research in this area would allow better informed decisions to be made, in order to determine whether CLA, a ruminant TFA which shows potential to be of benefit to health, and is present in human breast milk, is being correctly grouped within a category which is regarded as detrimental to health, and is kept to a minimum in infant formula milk.

Due to the current EFSA regulations, the best way to match CLA in infant formula milks manufactured from cow's milk to similar levels to those in human breast milk would be by increasing its content within the raw milk from which the infant formula milk is derived,

and by retaining at least some of the milk fat, rather than completely replacing it with a vegetable fat blend.

1.6 Sheep as a model for my investigation

Due to the possibility of adverse consequences for humans of dietary manipulations in early life, animal models are regularly used to study the effects of nutritional programming. Small animal models have a short gestational period, meaning research can be conducted quickly and at a comparatively low cost, but they have a number of disadvantages for investigating early life development. When compared with humans and larger animal models (e.g. sheep), small animals such as rodents are immature at birth. They also tend to be litter-bearing and, consequently, the position of fetuses in the uterus can affect the nutrients they receive and their birth weight[392-394]. These factors result in very different prenatal and postnatal environments in comparison to humans, who usually only bear one child.

Whilst no animal model is truly representative of humans, sheep have become a well-established model for BAT research in our laboratory. Both sheep and humans are born with a mature hypothalamic-pituitary-adrenal axis[395]. Lambs and human infants also have similar birth weights, and whereas the majority of humans give birth to one infant, sheep usually bear 1-3 lambs, depending on their breed. Whilst this makes them a better model than smaller animals that have larger litters, the variation of the intrauterine environment is a limitation when drawing comparisons between singleton offspring and multiple births. However, other aspects of the intrauterine environment are similar between sheep and humans, including organ development[396], which is key to fetal growth and placental transport of nutrients[396], specifically amino acid transfer[397]. Also, glucose transfer and metabolism, which has been shown to affect adipose tissue development[398].

Sheep are also a good model to use for a study investigating BAT, as BAT deposition occurs during the gestation period in sheep and humans, whereas rodents have little adipose tissue at birth[399], and the majority of their BAT deposition in occurs postnatally[113,400,401].

Another benefit of using sheep for this study is that, although sheep milk tends to be higher in protein and fat than human milk and lower in sugars[235], it has been shown that amongst human, sheep, pig, goat, cow and horse milk samples, sheep and human milk had the highest CLA content[402]. However, sheep milk CLA content also displayed the highest seasonal variation, so this needs to be taken into consideration when planning studies. A drawback of using sheep as a model in nutritional research is that sheep, unlike humans,

are herbivores and ruminants. Therefore their diet and the way they metabolise energy differs from humans[403]. However, in early life, which is my focus, both humans and lambs rely on milk as their sole source of nutrition.

Finally, for Chapter 4, where I study the effects of fatty acid supplementation on the milk composition, sheep milk is a reasonable model for cow's milk. The milk fatty acid profile of cows and sheep seem to show similar responses to season (Table 1.3) and fatty acid supplementation (Table 1.5). Therefore, my results could potentially inform further investigation regarding supplementing cow's diets, in order to alter the milk fatty acid profile of milk used to formulate infant formula milk.

1.7 Supplements used in my investigation

Dietary fatty acids have been used in numerous studies to alter the composition of ruminant milk, with the aim of improving it to benefit the health of adult humans[358,404,405].

Table 1.5 summarises the effects which canola and sunflower oil have on the milk composition in ruminants. My studies use canola oil (Chapters 3 and 4) and sunflower oil (Chapter 4) to induce changes in the fatty acid profile of ewes' milk. Maternal canola oil and sunflower oil supplementation have been shown to increase MUFAs and CLA, and decrease SFAs, in milk[406-409]. To my knowledge, no studies have investigated whether milk composition in early life, specifically its fatty acid profile, is capable of affecting the development of BAT and the amount of UCP1 in large mammals.

In addition to this, the supplementation of canola or sunflower oil appears to have a similar effect on milk fatty acid profile as grass feeding in the summer months (Table 1.3).

This enables me to investigate the potential effects that altering ruminant diet, to improve the milk fatty acid profile, could have on milk CLA content, and on the milk content of other fatty acids which vary with season, such as MUFAs and SFAs.

Supplementation of the lambs or ewes with CLA would have given me a clearer idea of the CLA-specific effects on offspring BAT. However, it was not used for two main reasons. Firstly, at the time of conducting the study, rumen-protected supplements of *cis*-9, *trans*-11 CLA were not available. Secondly, my study was never intended to be used as a model for lactating women, but rather as a model for the effects of altered milk composition on BAT in early life. This study utilised the ability to alter milk composition by maternal supplementation, which gave us the additional benefit of being able to assess the effects of

ruminant supplementation on milk fatty acid profile, and the resulting effects that these changes in milk could have in early life.

Study	Species	Supplement	Total fat	MUFAs	PUFAs	Total SFAs	Medium-chain SFAs	Long-chain SFAs			CLA			*TFAs*	Omega-3 FAs	Omega-6 FAs
								C14:0	C16:0	C18:0	Total CLA	*c*-9, *t*-11	*t*-10, *c*-12			
Mir *et al*.(1999)[410]	Goats	4/6% canola	↑	/	/	/	↓	/	↓	↑	↑	/	/	/	/	/
Okine *et al*.(2003)[406]	Goats	4/6% canola	↑	↑	↔	↓	↓	↓	↓	↑	↑	/	/	/	↓	↓
Welter *et al*.(2016)[407]	Cows	6% canola	↓	↑	↑	↓	↓	↓	↓	↑	/	↑	/	/	↑	↑
Rego *et al*.(2009)[408]	Cows	10% sunflower	↓	↑	↓	↓	↓	↓	↓	↑	↑	↑	↓	↑	↓	↓
Hervás *et al*.(2008)[409]	Sheep	6% sunflower	↔	↑	↔	↓	↓	↓	↓	↑	↑	↑	↑	/	/	/

↑ Increase with supplementation ↓ Decrease with supplementation ↔ No change / Not measured

Table 1.5 Summary of previous studies supplementing canola/sunflower oil and their effects on milk fatty acid profile.

MUFAs = monounsaturated fatty acids, PUFAs = polyunsaturated fatty acids, SFAs = saturated fatty acids, CLA = conjugated linoleic acid, TFAs = trans fatty acids, FAs = fatty acids.

1.8 Main hypothesis and aims

This chapter has discussed obesity and the potential of BAT to be used in its treatment and/or prevention, and the early life programming of obesity, specifically by early life nutrition. Fatty acids are known to activate *UCP1*, and I was particularly interested in the actions of CLA, a fatty acid which is known to be present in human breast milk, and which, whilst there is a paucity of published literature, appears to be present at lower levels, if at all, in infant formula milk.

The aim of my thesis is to investigate whether the composition of milk received in early life can affect BAT's thermogenic capacity (i.e. UCP1 abundance).

My main investigation set out to alter the fatty acid profile of milk by supplementing the maternal diet. The main hypothesis of my study is that:

- the fatty acid profile of milk received in early life can alter the development of BAT.

The chapters which follow were designed to investigate this hypothesis.

Chapter 3 is a description of and analyses of samples from a preliminary, feasibility study, 'Study A', which was conducted to trial the feasibility into the practicalities of running the investigation, such as whether the ewes would consume the diet, whether there were any adverse effects of the diet on ewes/lambs and which adipose tissue depots it would be possible to excise from the lambs at each time point. Samples were analysed once both Study A and Study B had been completed.

Chapter 4 details the first part of my main investigation, 'Study B', where mothers are supplemented with fatty acids during lactation in order to induce changes in their milk fatty acid profile.

Chapter 5, the second part of Study B, investigates the effects which the altered milk fatty acid profile has on the offsprings' adiposity and their BAT.

Chapter 6 investigates the content of CLA in a range of infant formula milk samples, to determine whether it is present and, if so, how its concentrations are comparable to those reported in human breast milk.

2 Materials and methods

In this Chapter, I will explain the basic scientific theory behind and the procedures of the experimental procedures used in this investigation.

My main work (Chapters 3, 4, 5) comprised of two separate studies, referred to throughout as 'Study A' (Chapter 3) and 'Study B' (Chapter4 and 5). This chapter details the methodology used, which, unless otherwise stated, was the same for both studies.

2.1 Procedures and legislation

All animal experimental procedures carried out in this thesis were conducted 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. The animal experiments were conducted by Professor Michael Symonds, assisted by Dr Mark Birtwistle, Dr Viv Perry, Dr Stuart Astbury (Study A) and Mr Graeme Davies (Study B). Both Studies A and B were funded by Danone Nutricia.

The laboratory-based techniques (with the exception of the milk and two of the plasma analyses) were conducted within the Department of Child Health, the University of Nottingham and the School of Veterinary Medicine, Sutton Bonington Campus between October 2011 and December 2015, under the supervision of Professor Michael Symonds and Professor Helen Budge.

All chemicals, reagents and laboratory procedures were assessed and implemented in compliance with the UK Health and Safety Executive's Control of Substances Hazardous to Health (COSHH, SI No. 1657, 1988) and Risk Assessment guidelines. Methods were followed as recommended by the relevant manufacturers or optimised by myself, Mr Mark Pope, Dr Ian Bloor or Mrs Victoria Wilson.

All materials were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise specified. Full details of all supplier names and addresses are given in Appendix 1 (Page 264).

2.2 Study design

Both studies were conducted in March-April (Study A in 2012 and Study B in 2013) and had the same design, where from the day of delivery until 28 days post-delivery, ewes were fed a control diet or a control diet supplemented with 3% canola oil. In the case of Study B, a further dietary group was added, this group was fed a control diet supplemented with 3% sunflower oil. Lambs were then mother fed until time of tissue sampling. The designs of Study A and Study B are shown in Figure 2.1 and Figure 2.2 respectively.

Aside from the additional dietary group, design and procedures for each study were the same, except where otherwise stated.

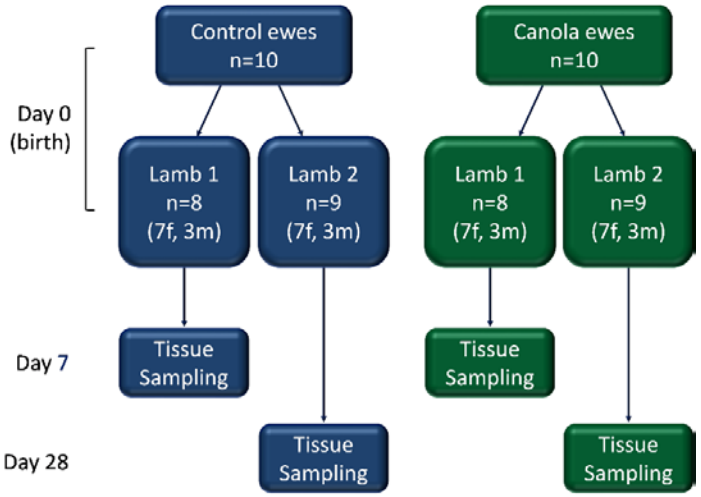

Figure 2.1 Summary of experimental design for Study A.

Control group ewes were fed a standardised diet, and canola group ewes were fed a standard diet +3% canola oil. All lambs were mother fed from birth until tissue sampling. The day numbers refer to days postpartum for ewes and days of age for lambs.

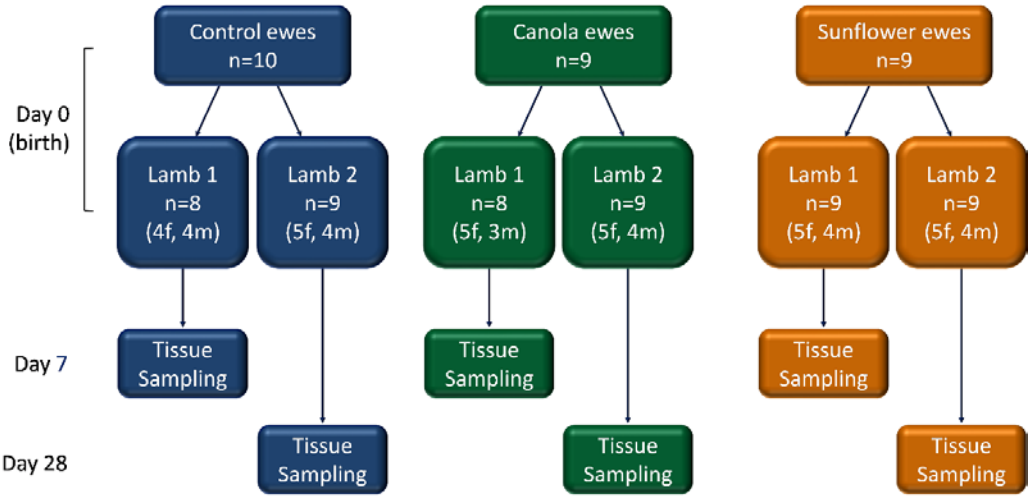

Figure 2.2 Summary of experimental design for Study B.

Control group ewes were fed a standard diet, canola group ewes were fed a standard diet + 3% canola oil and sunflower group ewes were fed a standard diet + 3% sunflower oil. All lambs were mother fed from birth until tissue sampling. The day numbers refer to days of age.

2.2.1 Animals

2.2.1.1 Study A

Twenty multiparous Zworval ewes were mated with 1 Charolais ram. One week before they were due to give birth, ewes were moved from a local farm (West Leake, Nottinghamshire) to the Melton Lane Complex on the University of Nottingham's Sutton Bonington campus.

Of the twenty ewes, fourteen gave birth to twins, five gave birth to triplets and one gave birth to quadruplets. Making a total of 47 lambs. When a ewe gave birth to more than 2 lambs, the extra lambs were removed, so that each sheep raised two lambs, leaving 40 lambs (28 females and 12 males). Immediately after delivery, ewes were allocated to one of two dietary groups controls (n=10) and canola supplemented (n=10).

2.2.1.2 Study B

28 multiparous Bluefaced Leicester cross Swaledale mule ewes were mated with one Texel ram.

Two weeks before they were due to give birth, they were transported from a commercial farm in Yorkshire to the Melton Lane Complex on the University of Nottingham's Sutton Bonington campus.

Of the twenty-eight ewes, twenty-three gave birth to twins and five to triplets making a total of 61 lambs (35 females and 26 males). Immediately after delivery, ewes were allocated to one of three dietary groups: Controls (n = 10); canola oil supplemented (n = 9) or sunflower oil supplemented (n = 9). Allocations were made so as to ensure, as far as possible, that each feeding group had an even distribution of offspring by sex.

Two female lambs died around the time of birth, leaving 59 surviving lambs (33 female and 26 male). One male lamb was excluded due to stunted growth. One lamb from each set of triplets (1M, 4F) was removed from its mother on the day after birth for tissue sampling, leaving its two siblings to be raised in the investigation. This left 53 remaining lambs (29 female and 25 male).

2.2.2 Environments

For both studies, upon arrival at the Melton Lane Complex, ewes were housed indoors, then moved to individual pens (2m x 2m) with their respective lambs after giving birth.

2.2.3 Diets

2.2.3.1 Ewes

For both studies, the experimental intervention started from the birth of the lambs (day 0).

In both Study A and Study B, there was a control and canola oil supplemented group. For Study B, there was an additional, sunflower oil supplemented, group. The diets were as follows:

- controls - 1.5kg of standard concentrate (Ewe 18 mix, Manor Farm Feeds, Oakham, Leicestershire, UK) and 1.5kg roughage.
- canola group - 1.5kg concentrate which contained 3% canola oil (Ewe 18 mix, +3% canola oil, Manor Farm Feeds), along with 1.5kg of roughage
- sunflower group - 1.5kg concentrate which contained 3% sunflower oil (Ewe 18 mix + 3% sunflower oil, Manor Farm Feeds), along with 1.5kg of roughage.

The concentrate was formulated for pregnant and lactating ewes, and was sufficient to fulfil energy requirements[411]. Constituents of the concentrates are detailed in Table 2.1.

The 3% addition of canola/sunflower oil meant that the total fat was 6% in the intervention groups compared with 3% in controls (Table 2.1). Both groups had free access to water.

Constituent	Control (%)	Canola (%)	Sunflower (%)
Canola oil	0	3	0
Sunflower oil	0	0	3
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

Table 2.1 Constituents of concentrate feed by dietary group.

2.2.3.2 Lambs

All lambs were mother fed from birth until they were tissue sampled (day 7 or 28).

2.2.4 Blood sampling

In study B, unfasted blood samples were taken from all the ewes on days 7 and 28 and from lambs on the day of their tissue sampling (day 7 or day 28). Up to 20ml of blood was taken from the jugular vein with a 10ml syringe in two extractions. Blood was transferred into a 10 ml ethylenediaminetetraacetic acid (EDTA) tube (Vacutainer; BD, Plymouth, Devon, UK) and a 9ml lithium heparin tube (S-Monovette; Sarstedt, Nümbrecht, Germany), which were transferred to ice immediately and centrifuged at 4°C, 1200g for 15 min. The plasma was then dispensed into 2ml cryovials and snap frozen in liquid nitrogen, for storage at −80°C. One sample per animal was shipped on dry ice to Professor Duane Keisler, University of Missouri, USA, for analysis of IGF-1 and leptin, as previously described[412]. One sample per animal was analysed by myself and Dr Nigel Kendall at the University of Nottingham School of Veterinary Medicine, Sutton Bonington Campus (detailed in Section 2.6).

2.2.5 Milk sampling

For both studies, milk samples were taken by hand from ewes on day 7 and 28. Milk was transferred into two sterile 15ml tubes (Greiner Bio-One, Gloucester, UK). The samples were then stored at -80°C until being shipped on dry ice to Tamas Marosvolgyi at the University of Pécs, Hungary for analysis of the milk fatty acids, using high-resolution capillary gas-liquid chromatography, as previously described[413].

2.2.6 Body weight measurements

Lamb body weight (kg) was measured for lambs on day of birth (day 0), day 7 and day 28. Ewe body weights were taken on days 7 and 28 for Study B.

2.2.7 Tissue sampling

For both studies, one lamb from each ewe was tissue sampled at 7 (± 1) days of age and the other at 28 days of age (± 3) days of age.

The lambs were humanely euthanased by an injection of an overdose of barbiturate (200 mg kg^{-1} body weight of pentobarbital sodium; Euthatal; Merial Animal Health, Harlow, Essex, UK) into the jugular vein. Lambs were then dissected, and tissues and organs were removed, weighed and snap-frozen in liquid nitrogen before being stored at −80°C until subsequent analysis. Samples from each adipose tissue depot were also preserved in 10% formalin (3.45% formaldehyde, 0.9 % sodium chloride) for subsequent histological analysis. Dissections were carried out by Professor Mike Symonds, Dr Mark Birtwistle and Dr Viv Perry and assisted by Dr Stuart Astbury (Study A) Mr Graeme Davies (Study B).

2.3 Tissue gene expression analysis

The following procedure was used for the gene expression analysis on the perirenal adipose tissue of lambs in Studies A and B.

Prior to all tissue and molecular work, the work benches and instruments were cleaned with RNase ZAP® (Ambion, CA, USA) and 70% ethanol. Where necessary, experiments were performed on ice. In order to prevent contamination, experimental work was carried out using autoclaved equipment and filtered pipette tips.

2.3.1 Ribonucleic acid extraction

Ribonucleic acid (RNA) can be extracted from tissue using an RNA extraction kit. First, proteins are dissolved and RNase activity is inhibited by homogenising the sample in a monophase solution containing phenol and guanidine thiocyante[414]. The dissolved proteins, DNA and RNA are separated by adding chloroform[415]. Ethanol is then added to the RNA solution, which provides the correct binding conditions, allowing the RNA to bind to the membrane of the RNA column in which the solution is placed.

The RNA concentration can be determined by using spectrophotometry, which estimates RNA concentration by measuring the solution's absorbency relative to that of pure nucleic acids, (nucleic acids absorb ultraviolet (UV) light at a wavelength of 260nm). Protein contamination can be determined by calculating the ratio of 260nm:280nm[416] (protein absorbs UV light at 280nm).

RNA was extracted from the tissue using RNeasy plus kit (Qiagen, Crawley, UK) in line with the following procedure.

2.3.1.1 Procedure

For RNA extraction, 250mg of adipose tissue was thawed in 1ml of Tri-reagent (Sigma-Aldrich) and homogenised in a Dispomix® closed system homogeniser (Thistle Scientific Ltd. Glasgow, UK). The homogenised samples were then placed in a 37°C water bath for 2 minutes in order to break down the lipids. The homogenate was vortexed for 30 seconds and centrifuged at room temperature for 5 minutes at 1800g. The lower phase of the sample was carefully transferred into a sterile 2ml Eppendorf tube and 200µl of chloroform added and the tube inverted to mix the solution.

Following this, samples were incubated at room temperature for 10 minutes before being centrifuged at 4°C for 15 minutes at 18000g. The upper aqueous phase of the solution was carefully aspirated onto a genomic DNA (gDNA) eliminator column (contained in the kit) for each sample, and samples centrifuged at 4°C for 30 seconds at 18000g. An equal volume of ethanol was added to the samples and they were thoroughly pipette mixed. Next, samples were pipetted into an RNeasy spin column (contained in the kit) then centrifuged at 4°C for 15 seconds at 18000g and the flow-through discarded.

Following this, 700µl of RW1 (premade buffer, contained in the kit) was added to each sample and all samples were centrifuged at 4°C for 15 seconds at 18000g, and the flow-through discarded. Then 500µl of RPE buffer (premade buffer, contained in the kit) was added to each sample and the samples were centrifuged at 4°C for 15 seconds at 18000g, and the flow-through discarded. A further 500µl of RPE buffer was then added to each sample, and then all samples were centrifuged again at 4°C for 2 minutes at 18000g, with the resulting flow-through being discarded.

Following this, RNeasy spin columns were transferred to a new 1.5ml tube and centrifuged at 4°C for 1 minute at 18000g to remove all RPE buffer. Each RNeasy spin column was then

transferred to a new 1.5ml tube and 50µl of nuclease free water was added to each column. Columns were centrifuged at 4°C for 1 minute at 18000g, with the elute being added back onto the column which was centrifuged again 4°C for 1 minute at 18000g, to increase the RNA elution yield.

RNA concentration was determined using a Nanodrop® spectrophotometer (Thermo Scientific, DE, USA).

Although the RNeasy plus kit contains constituents to remove genomic DNA (gDNA), to ensure prevention of any contamination from any residual gDNA, extracted RNA was treated with a DNase kit, RQ1 RNase-Free DNase (Promega, Southampton, UK). The amount of RNA required to make a solution of 1µg of RNA was calculated for each sample, this was added to an Eppendorf tube and made up to a total of 8µl using nuclease free water. Then, 1µl of RQ1 DNase (Promega) and 1µl of RQ1 DNase 10X reaction buffer (contained within the kit) was added to each RNA sample. Samples were then centrifuged at room temperature at 18000g for 30 seconds, and placed in a thermocycler (TouchGene Gradient, Techne Ltd., Cambridge, UK), on a pre-set cycle. After 30 minutes, the cycle was paused, samples removed and 1 µl of DNase stop solution was added to each tube to stop the DNase reaction. The samples were then returned to the thermocycler, and the program resumed. Table 2.2 details the times and temperatures for the gDNA elimination protocol.

Temperature (°C)	**Time (minutes)**
37	30
Pause	
65	10
8	hold ∞

Table 2.2 Details of the pre-set cycle samples are run on for the gDNA elimination step

When the program was complete, samples were removed from the thermocycler and reverse transcription carried out, where 10µl of each RNA sample was aliquoted, ready to undergo reverse transcriptase-polymerase chain reaction (RT-PCR). The remaining RNA was all stored at -80°C until required.

2.3.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) allows the isolation, amplification and subsequent analysis of a known DNA sequence, using a pair of complementary oligonucleotide primers, to identify the beginning and end of the region of interest, in an enzymatic reaction[417]. The amplification takes place by repeated cycles of heat denaturation, where, upon denaturation the target DNAs double helix strand separates. The primers then anneal to the 3' terminus of each strand and an enzymatic reaction extends the annealed primers. This is illustrated in Figure 2.3. Each cycle doubles the length of the DNA, leading to an exponential amplification of the DNA, of approximately 2^n (n = number of cycles) [418].

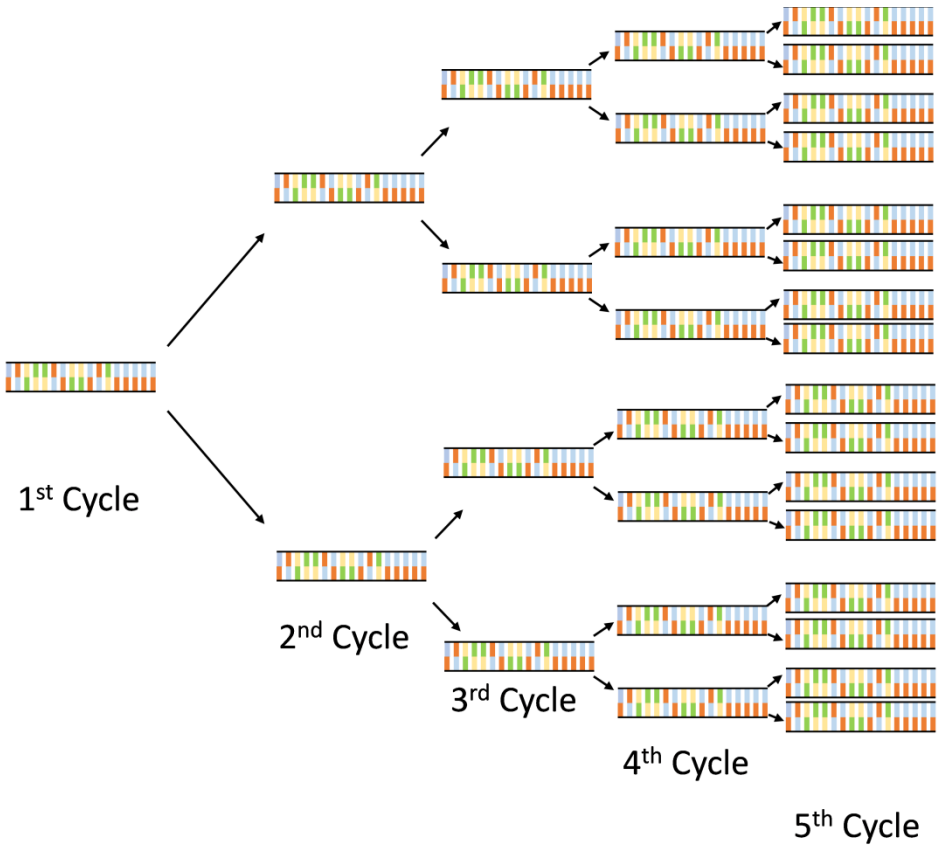

Figure 2.3 Illustration of the amplification which occurs during the polymerase chain reaction.

The accuracy of quantitative polymerase chain reaction (qPCR) is governed by the design of primers, which need to be specific to the region of interest, as elongation and amplification will only occur to the DNA sequence to which the primers have annealed. If a set of primers are poorly designed, the incorrect sequence could be amplified, or primer pairs could bind to one another, affecting results. The guidelines for optimal PCR primers are described by Butler[417] as:

- primers 70-250 base pairs in length
- a melting temperature (Tm) of 55-72°C (less than 5°C difference between forward and reverse primers
- a guanine/cytosine content of 40-60%
- less than three continuous self-complementary bases
- less than three continuous bases which complement the other primer
- less than 4 identical continuous bases
- a distance of fewer than 2000 bases between primers on the target sequence

2.3.3 Reverse transcriptase PCR

RT-PCR is a technique which reversely transcribes RNA into its complementary DNA, using a DNA primer and a reverse transcriptase enzyme. This needs to be carried out before classical polymerase chain reaction (PCR) in order to transcribe it into a double-stranded complementary DNA (cDNA), as PCR works by amplifying a double-stranded molecule (DNA)

A random primer binds to the template mRNA and the RT enzyme extends the sequence, creating cDNA (Figure 2.4), which can then undergo classical PCR, to be amplified.

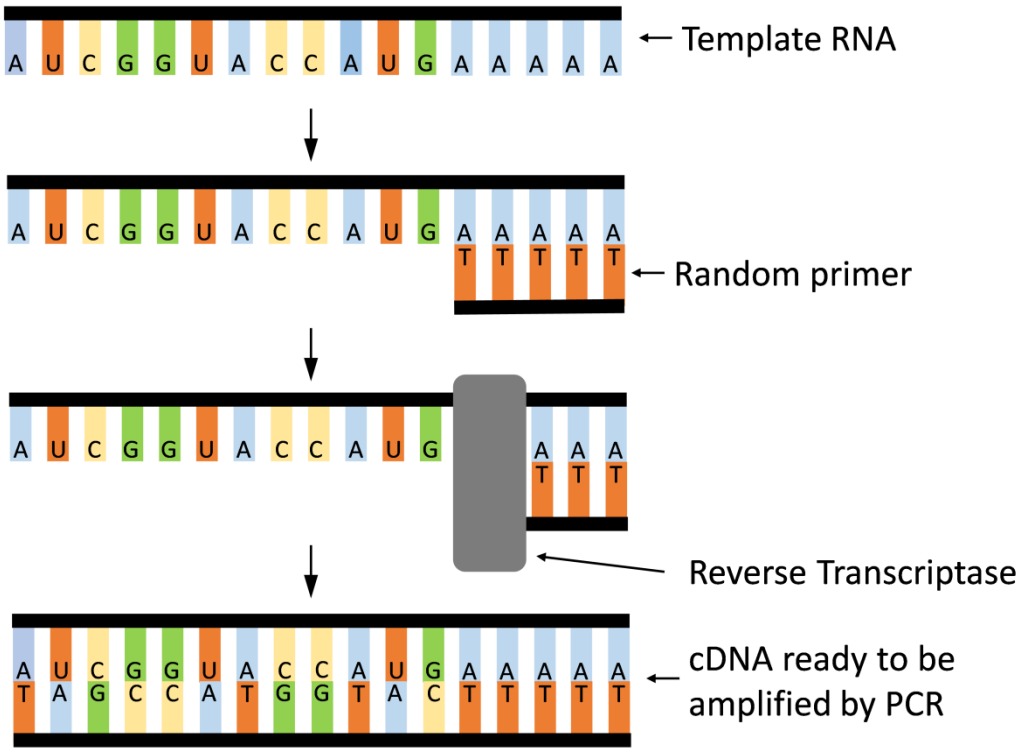

Figure 2.4 Illustration of reverse transcriptase polymerase chain reaction.

A random primer anneals to template RNA, and then a reverse transcriptase enzyme elongates the sequence, creating a double-stranded cDNA, which can be amplified by polymerase chain reaction (PCR).

2.3.4 Agarose gel electrophoresis and DNA extraction

Running the DNA on an agarose gel allows confirmation of the presence of the gene of interest in the sample, and also determines whether there is any contamination in the samples (by using a negative control). If there is more than one band or a band in the negative control lane, that is an indication that there is contamination, or primer dimerization. 'Primer dimers' occur when complementary bases within the primers attach to each other, resulting in nonspecific amplification.

The negative charge of the DNA phosphate backbone allows the molecule to migrate towards the positive electrode. Different DNA fragments move at different speeds, depending on their size. This process thus separates different sizes of DNA fragments. Ethidium bromide is added to the agarose gel due to its fluorescent properties, which allow detection of the DNA (to which it binds) under a UV light.

2.3.5 Quantitative polymerase chain reaction

In order to get a more precise measure of the quantities of a particular gene within a sample, quantitative polymerase chain reaction (qPCR) is used. This allows for nucleic acid analysis to be achieved in real-time, where a DNA or mRNA sequence can be detected and quantified at the same time *in vitro,* without the need for additional procedures such as gel electrophoresis, thereby saving time and money, in addition to reducing the risk of contamination[419].

Like in classical PCR, during qPCR, the DNA sequence is amplified, with the addition of a fluorogenic chemical reagent (a fluorescent dye), which fluoresces when bound to DNA. The results are based on the measurements of fluorescence signals, which increase with increased DNA quantities[420]. As the fluorescent dyes emit a basal level of fluorescence, the qPCR instrument will only detect the fluorescence when it exceeds a certain threshold (to eliminate background fluorescence). This threshold is referred to at the cycle threshold (C_t). This is the parameter used for quantification, to give the amount of target template present within the initial sample[420]. SYBR Green is a commonly used fluorescent dye, which binds to double-stranded DNA and fluoresces. This has the desired effect, when it binds to the intended target, however, it is non-specific, which means that it will bind to any DNA present, making it important to ensure gDNA and any other contamination is eliminated, and that primers are designed which minimise the risk of primer dimers.

There are advantages in using SYBR green, in that it can be used with any gene, making it time and cost efficient. It is also particularly advantageous in my animal model – the sheep, whose genome has not yet been fully annotated, and therefore designing probes (specific) is challenging. In order to help eliminate the issues associated with SYBR green being non-specific, it is usually possible to detect any non-specific amplification by analysing melt curves. A melt curve is generated at the end of a SYBR Green qPCR reaction, where the samples are heated and the DNA is denatured, which causes the fluorescence to start to decrease. This decrease is quantified, and if there is a single product there should be a single peak in the curve[421] (Figure 2.5), multiple peaks indicate the presence of secondary products.

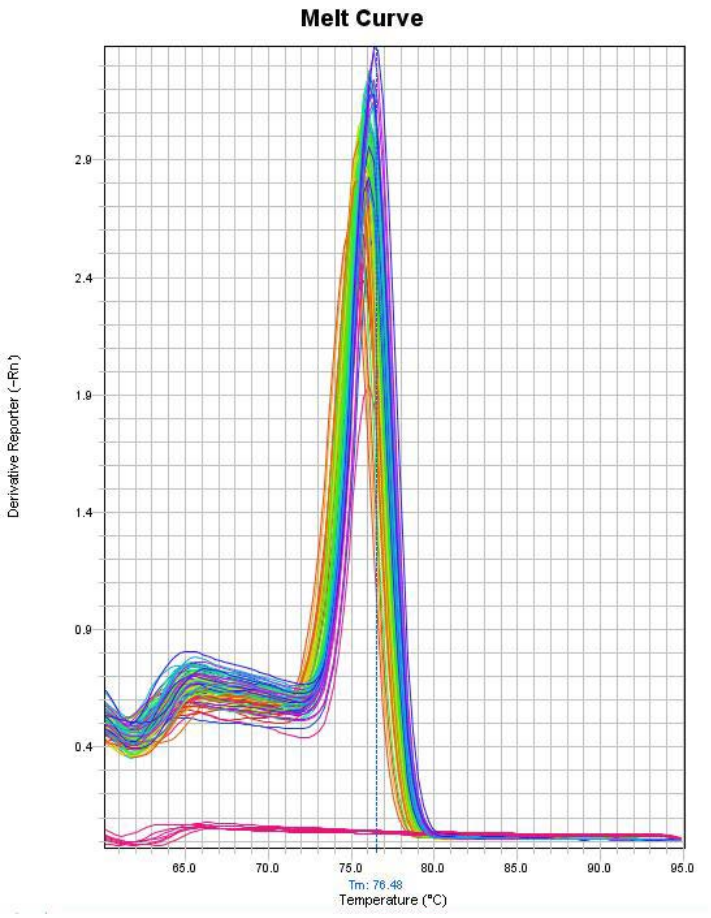

Figure 2.5 Representative melt curve from qPCR.

The melt curve shows the amplification of a single product. The pink coloured lines represent the negative controls.

Target gene relative quantities were divided by the geometric mean of the most stable reference genes (Section 0, Page 64) to produce an output value for analysis. The geNorm method (geNorm software v3.5, Primer Design, Southampton, UK) was used to determine the relative expression of target genes, taking into account the individual efficiency of each assay.

The exponential amplification within the PCR reaction is graphically portrayed by the instrument, showing the increase in fluorescent signal as a sigmoidal, "S" shaped, curve (Figure 2.6). This can be used to compare signals between samples run within the same experiment.

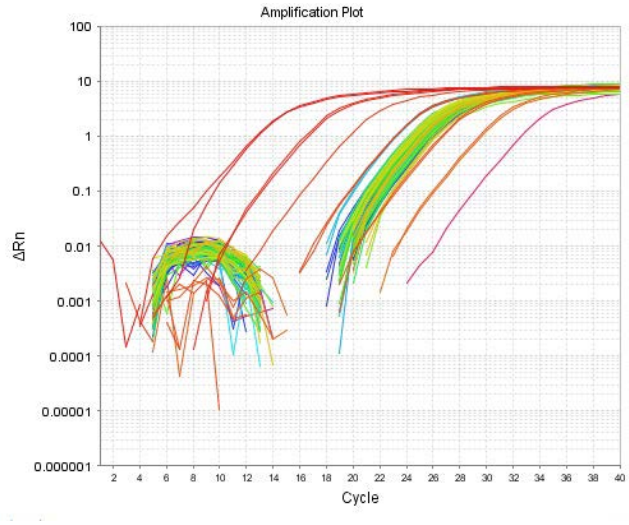

Figure 2.6 Example of an amplification plot from a qPCR instrument.

Fluorescent signal is increased exponentially with amplification. A plateau is reached when the reagents are depleted.

By preparing a standard curve (a series of dilutions) using known quantities of DNA, it allows comparisons to be made with the unknown samples in order to determine their DNA quantity. The log concentrations (ng/µl) of the standard curve dilutions are plotted against the cycle number in which the C_t was reached and a straight line equation is calculated (Figure 2.7). From this, the reaction efficiency (E) and the correlation coefficient (R^2) can be determined. Efficiency can be calculated using the following formula, efficiency = $10(^{-1/gradient})-1)$[422]. This is derived from the equation describing PCR amplification[423].

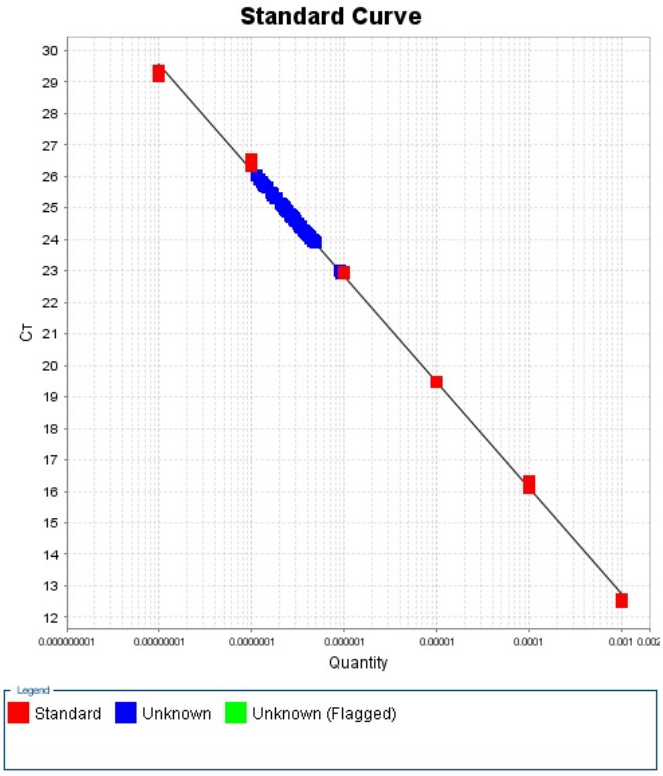

Figure 2.7 Example of a standard curve from qPCR.

Log concentrations (ng/µl) of the standard curve dilutions are plotted against the cycle at which the cycle threshold was reached. From this, the correlation coefficient and reaction efficiency are calculated.

2.3.6 The use of reference genes

In order to be able to make adequate comparisons between qPCR measurements, it is of the high importance that the data is normalized[424]. This controls for variation in the total amount of RNA present for each sample, and also for any variability, whether it be biological or methodical.

In order to correctly use reference genes, the selected genes must be universally and stably expressed in the samples being analysed[424]. This allows variances which have occurred due to experimental error to be identified and corrected for.

The appropriate reference genes to use vary greatly between samples, and using the wrong reference gene could provide false results[425]. Therefore it is imperative that the correct reference gene is selected. In order to quantify gene expression, the expression of a gene of interest is compared to the geometric mean expression of selected reference genes in the same sample.

2.3.7 Reverse transcriptase polymerase chain reaction procedure

The procedure was carried out as follows using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA).

DNase-treated RNA samples were placed on ice. 12.5µl of 2X RT buffer (contained within the kit) was added to each sample, along with 0.25µl of nuclease free water and 1.25µl of 20X RT Enzyme Mix (contained within the kit).

Two negative controls were also prepared; a no template control (NTC) which contained the RT buffer, the RT enzyme mix, but nuclease free water in place of the sample and a no reverse transcriptase control (NRTC) which contained the RNA sample, the RT buffer but nuclease free water in place of the RT enzyme (detailed in Table 2.3).

	Sample (µl)	NTC (µl)	NRTC (µl)
RNA	11	0	9
RT buffer	12.5	12.5	12.5
RT enzyme mix	1.25	1.25	0
Nuclease free water	0.25	0.25	1.5

Table 2.3 Details of the reagents and quantities used to prepare the samples, no template control (NTC) and no reverse transcriptase control (NTRC).

The samples were then centrifuged at room temperature for 1 minute at 5000g and placed into the PCR machine on a pre-set cycle (described in Table 2.4). After completion of RT, samples were diluted by 1 in 8 to produce a 200µl stock of cDNA solution for each sample. Dilutions were made with a 10 ng μl^{-1} solution of yeast transfer RNA (tRNA). This acts as a carrier molecule and competes with binding sites in the plastic Eppendorf tube, preventing binding of the nucleic acid of interest, improving the signal in qPCR. After dilution, samples were stored at -20°C, until further analysis.

Temperature (°C)	Time (minutes)
37	60
95	5
8	Hold ∞

Table 2.4 Details of the pre-set cycle for polymerase chain reaction.

2.3.7.1 Classical polymerase chain reaction procedure

A sterile Eppendorf tube was labelled for each sample and for one NTC. A sterile 1.5ml Eppendorf tube was labelled for the master mix, which was prepared to contain the correct quantities of Thermo-Start® PCR master mix (ABgene LTD., UK), forward and reverse primers (diluted to 1:40). Volumes (Table 2.5) are calculated allowing for pipetting errors and pipette tip wastage by adding an extra 2µl of each reagent. Then, 18µl of the master mix was added to each Eppendorf tube alongside 2µl of the corresponding cDNA sample or in the case of the NTC, 2µl of nuclease free water.

Reagent	Per sample (µl)	Master mix (µl)
Stock PCR master mix	10	10 (x+2)
Forward primer (1:40 dilution)	4	4 (x+2)
Reverse primer (1:40 dilution)	4	4 (x+2)
cDNA	2	
Total	20	18 (x+2)

Table 2.5 Details of reagents and quantities used for polymerase chain reaction (PCR) samples.

All samples were centrifuged at room temperature for 1 minute at 10000g and placed into the PCR machine (TouchGene Gradient, Techne, Cambridge, UK) on a pre-set program (Table 2.6).

Temperature (°C)		Time
94		30 seconds
40 cycles	94	30 seconds
	60	30 seconds
	68	1 minute
68		5 minutes
8		Hold∞

Table 2.6 Details of the classical polymerase chain reaction (PCR) standard program.

2.3.7.2 Agarose gel electrophoresis and DNA extraction procedure

The PCR products were run on an agarose gel using the gel electrophoresis procedure. A 2% (w/v) agarose gel was prepared in a conical flask by mixing 1g of agarose powder with 50ml of 1 x tris (hydroxymethyl) aminomethane base (Tris), acetic acid and EDTA buffer. This was heated and 2.5µl of 10mg/ml ethidium bromide was added once the gel had cooled slightly. The gel was then poured into a taped cassette and the required number of combs inserted to create wells. The gel was left to set for 10 minutes, then the combs removed and the gel placed in an electrophoresis tank filled with 1x Tris-Acetate-EDTA buffer to cover the gel.

Samples were then prepared by adding 4µl of a 1 in 6 dilution of loading dye to each sample and thoroughly pipette mixing. To the first well, 5µl of 100 base pair (bp) DNA ladder (Fisher Scientific Ltd, UK) was added, followed by each of the samples in the subsequent wells. On completion of this the tank was connected to a power unit set at 80V and run for 30 minutes, or until the dye had progressed a suitable distance. Following this, the gel was imaged using a Fuji film luminescent image analyser LAS-3000 (Raytek, Sheffield, UK). The bands were cut and transferred into a labelled, sterile 1.5ml Eppendorf tube, and stored at -20°C until DNA extraction.

DNA was extracted from the gel using a QIAquick gel extraction kit. Gel bands were weighed and mixed with 3 volumes of Buffer QG (premade buffer contained in the kit) to 1 volume of gel, in a 1.5ml Eppendorf tube. The tubes were then incubated at 50°C for 10 minutes being vortexed every 2-3 minutes until the gel had dissolved. Following this 1 gel volume of isopropanol was added to each sample, for DNA elution. The samples were then transferred to a QIAquick spin column (contained within the kit) which was inserted into a sterile 1.5ml Eppendorf tube and placed in a microcentrifuge at room temperature for 1 minute at 10000g. The flow-through was discarded and 500µl of buffer QG was added to the columns and they were centrifuged at room temperature for 1 minute at 10000g. 750µl of buffer PE (premade buffer, contained in the kit) was added to the columns, which were left to stand for 2-5 minutes before being centrifuged at room temperature for 1 minute at 10000g. The flow-through was discarded and the columns centrifuged for a further 1 minute at room temperature at 10000g. The columns were then placed into a new sterile 1.5ml Eppendorf tube and 30µl of Buffer EB (premade elution buffer, contained in the kit, 10mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane and the columns were left to stand for 1 minute then centrifuged at room temperature for 1 minute at 10000g.

DNA concentration was determined using a Nanodrop® spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.3.8 Quantitative PCR procedure

A standard curve was used to make it possible to calculate the efficiency of the reaction. For each gene, DNA of a known concentration, extracted from the agarose gels was used to make up a standard curve. A serial dilution was made in 9 Eppendorf tubes, with concentrations ranging from 0.1 ng/µl to 1 x 10^{-8} ng/µl.

A master mix was prepared, containing SYBR Green master mix, a forward primer and a reverse primer and 7µl of master mix and was carefully pipetted into each well of a sterile 96-well plate. Then, 3µl of cDNA was added to the wells of the samples and 3µl of known concentration standards was added to the standard curve wells. In the case of the negative control, 3µl of nuclease free water was added in the place of cDNA. Quantities of sample and reagents are shown in in Table 2.7. All samples were pipetted in duplicate.

Reagent	Per sample (µl)	Master mix (µl)
SYBR Green master mix	5	5(2x+2)
Forward primer	1	(2x+2)
Reverse primer	1	(2x+2)
Subtotal for master mix	**7**	**7 (2x+2)**
cDNA	3	
Total sample volume	**10**	

Table 2.7 Details on reagents and quantities used for quantitative polymerase chain reaction (qPCR) samples.

Once samples and master mix had been added to each well, the top of the plate was sealed with a plastic film, placed in the PCR machine and run on a pre-set program (detailed in Table 2.8).

Stage		Temperature (°C)	Time (seconds)
Activation of DNA polymerase		95	20
Cycling stage			
40 cycles	**Step 1**	95	3
	Step 2	60	30
Melt curve stage			
	Step 1	95	15
	Step 2	60	60
	Step 3	95	15

Table 2.8 Details of the real-time polymerase chain reaction standard program.

2.3.8.1 Selection of reference genes

Seven potential reference genes to be used for ovine perirenal adipose tissue samples were identified, these were *18S*, *YWHAZ*, *IPO8*, *RPL19*, *GAPDH*, *SDHA* and *RPLP0*.

The expression of these reference genes in the mRNA of all tissue samples was analysed by qPCR. The geNorm algorithm[426] was then used to calculate the stability (given as an M value) for each reference gene, using pairwise variation. The two reference genes with the lowest M values, *IPO8* and *RPO,* were used to normalise all genes of interest in my investigations.

2.3.9 Primers for qPCR

Primer sequences used in this study were either from published studies or designed in house using the online primer design tool NCBI Primer-BLAST[427]. This software checks primers for the risk of amplification of unintended targets, allowing those with few, or no unintended targets to be selected. In order to reduce the risk of amplification of gDNA, primers, where possible, were designed to span an exon-exon junction, ideally with one primer flanking the exon-intron boundary. This was not possible for all primers, for example, *RIP140*, which has only one exon. Primers designed in house were sequenced and compared against the bovine/ovine genome, in order to confirm that they were, indeed, specific to the intended target gene. Sequences for primers used in this investigation can be found in Table 2.9.

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Gene	Gene function	Reference or NCBI number	Forward primer sequence	Reverse primer sequence	Amplicon length (base pairs)
IPO8	Reference gene	Pope *et al*. (2014)[428]	GCCCTTGCTCTTCAGTCATT	GTGCAACAGCTCCTGCATAA	99
RPLP0	Reference gene	Robinson *et al*. (2007)[429]	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	227
YWHAZ	Reference gene	NM_001267887.1	TGTAGGAGCCCGTAGGTCA	TTCTCTCTGTATTCTCGAGCCATCT	102
SDHA	Reference gene	Garcia-Crespo *et al*. (2005) [430]	CATCCACTACATGACGGAGCA	ATCTTGCCATCTTCAGTTCTGCTA	90
18s	Reference gene	Williams *et al*. (2007)[431]	GATGCGGCGGCGTTATTCC	CTCCTGGTGGTGCCCTTCC	126
UCP1	BAT/thermogenesis	NM_001166528.1	GGGCTTTGGAAAGGGACTA	CAGGGCACATCGTCTGCTAAT	128
ADRβ3	BAT/thermogenesis	NM_174232.2	AGAGAGCGAAAACCCACTTG	TTCCTGCGGACTTTCTTAGG	209
CIDEA	BAT/thermogenesis	NM_001083449.1	AAGGCCACCATGTACGAGAT	GGTGCCCATGTGGATAAGACA	138
DIO2	BAT/thermogenesis	Pope *et al*. (2014)[428]	AGCCGCTCCAAGTCCACTC	TTCCACTGGTGTCACCTCCT	175
Continued overleaf					

Gene	Gene function	Reference	Forward primer sequence	Reverse primer sequence	Amplicon length (base pairs)
PRDM16	BAT/thermogenesis	XM_003583245.1	TGGCAGCTGGCTCAAGTACA	CGGAACGTGGGCTCCTCATC	198
PRLR	BAT/thermogenesis	Pope *et al*. (2014)[428]	CTCCACCCACCATGACTGAT	CTCCACCCACCATGACTGAT	169
Leptin	White adipose tissue	Pope *et al*. (2014)[428]	CCAGGATGACACCAAAACC	TGGACAAACTCAGGAGAGG	140
TCF21	White adipose tissue	XM_004011346.3	ATCCTGGCCAACGACAAGTA	TCAGGTCACTCTCGGGTTTC	94
HOXC9	Brite adipose tissue	XM_002704244.2	GACCTGGACCCCAGCAAC	GCTCGGTGAGGTTGAGAAC	175
SHOX2	Brite adipose tissue	NM_001205527.1	CGCCTTTATGCGTGAAGAAC	TTGGCTGGCAGCTCCTAT	142
ADIPOQ	adipogenic/metabolic	Pope *et al*. (2014)[428]	ATCAAACTCTGGAACCTCCTATCTAC	TTGCATTGCAGGCTCAAG	232
FABP4	adipogenic/metabolic	Pope *et al*. (2014)[428]	TGAAATCACTCCAGATGACAGG	TGGTGGTTGATTTTCCATCC	98
PPARγ	adipogenic/metabolic	Pope *et al*. (2014)[428]	GACCCGATGGCTCCGTTA	TGAGGGAGTTGGAAGGCTCT	145
RIP140	adipogenic/metabolic	Pope *et al*. (2014)[428]	CGAGGACTTGAAACCAGAGC	TCTTAGGGACCATGCAAAGG	179

Table 2.9 Primer sequences for reference genes and genes of interest.

Primers were either from previously published papers or developed in house.

2.4 Western blotting

Western blotting is a commonly used technique to detect the presence of specific proteins, using antibodies[432]. After a tissue sample has had its protein, or fractionated organelle protein (in the case of my investigation, the mitochondrial protein) extracted, its concentration is measured using the bicinchoninic acid (BCA). The samples are then dissociated by heating alongside sodium dodecyl sulphate (SDS, which gives the proteins a negative charge[432] and β – mercaptoethanol, which breaks down the disulphide bonds linking the amino acids, disrupting the structure of the proteins. The samples are then loaded onto a gel and separated by electrophoresis, with the negative charge which the proteins have acquired from the SDS causing them to migrate towards the positive electrode, according to their size. The use of ladders of known protein sizes enables the size of the samples to be checked, to ensure it is the protein of interest. The use of loading dyes allows the movement of the samples through the gel to be visually monitored.

After the samples have been separated through electrophoresis, they can be blotted onto an electrostatic nitrocellulose membrane, using semi-dry electroblotting methods. A membrane is placed directly onto the gel, and buffer soaked filter paper is placed on either side of the gel-membrane sandwich, which is placed in a semi-dry blotter. An electric charge is then passed through, which causes the proteins on the gel to be transferred to the membrane. Following the protein transfer, the membrane is stained with a reversible dye in order to determine that the protein has been transferred evenly.

Membranes are then placed in a blocking solution containing a non-relevant protein, which prevents the non-specific binding of antibodies. Following this, it is exposed to the primary antibody which is specific to the protein of interest. The primary antibody can either be labelled with a detectable product (direct detection) or if a membrane has been exposed to an unlabelled primary antibody, a labelled secondary antibody can be used (indirect detection), shown in Figure 2.8. A secondary antibody binds to the primary antibody. The secondary antibody used in my investigation was labelled with horseradish peroxidase (HRP), and is an enzymatic tag which produces a detectable signal[433] when exposed to luminol. This signal is proportional to the abundance of the protein of interest, allowing its quantification using densitometry software.

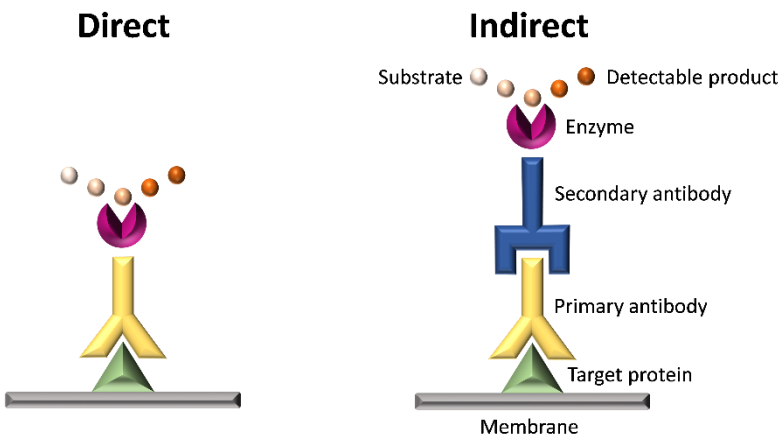

Figure 2.8 The principles of direct and indirect antibody action.

The direct method uses a specific primary antibody which produces a detectable product upon binding with the target protein. The indirect method uses a secondary antibody, which bonds with the primary antibody and produces a detectable product.

2.4.1 Western blotting mitochondrial preparation procedure

To prepare the mitochondria, 1ml of mitochondrial homogenisation buffer (10mM TRIS solution, 250mM sucrose solution, 1mM EDTA solution, pH 7.4) was added to 200mg of each tissue sample and homogenised in a Dispomix® closed system homogeniser. The homogenate was then centrifuged at room temperature at 3220g for 1 minute and all the homogenate from each sample transferred into 3 separate 1.5ml Eppendorf tubes. 100 µl of homogenisation buffer was added to each Dispomix® tube to 'wash' out any remaining tissue. This solution was then added to the corresponding Eppendorf tubes. Samples were then further homogenised using a hand-held Potter-Elvehjem homogeniser.

The homogenate for each sample was transferred into one Eppendorf tube and centrifuged at 4°C at 1500g for 10 minutes. The supernatant was transferred into another Eppendorf tube and centrifuged at 4˚C at 13000g for 60 minutes. Following this, the supernatant was removed and the remaining mitochondrial pellet was re-suspended in 100µl of homogenisation buffer.

2.4.2 BCA protein determination

In order to determine the mitochondrial protein concentration, the BCA assay was used. This assay relies upon the colorimetric reactions between proteins, BCA and copper sulphate[434,435]. When mixed in the solution, peptide bonds present in the proteins reduce Cu^{2+} to Cu^{1+} ions when incubated at room temperature. The BCA reagent then combines

with Cu^{1+} ions which forms a BCA-Cu+ complex, that is purple in colour[434]. The colour change (from a green to purple) is proportional to the amount of protein present in the sample, and absorbance can be measured at 562nm. The unknown concentrations can then be referenced against a standard curve, made up of known concentrations.

2.4.2.1 BCA assay procedure

For this assay, 50mls of 'reagent A' was made, using 1% bicinchoninic acid, 2% sodium carbonate, 0.16% sodium tartrate and 0.4% sodium hydroxide, which was made up to volume using distilled water, and made to pH11.25 using 10% sodium hydroxide. Then, 50ml of 'reagent B' was made up using 4% copper sulphate with distilled water. Reagents A and B were then mixed, to the ratio of 100ml: 2ml, to produce 'reagent C', which was stored at 4°C.

Standards were made using 8 concentrations of bovine serum albumin (BSA) ranging from 1.0, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 mg/ml, made up with 0.9% saline. 2.5µl of each unknown sample was diluted by 1:10 in 0.9% saline, to make up a total volume of 50µl. Some samples which had a high mitochondrial protein (above the upper detectable limit) were further diluted, to a final dilution of 1:20.

Following this, 10µl of each standard, sample or saline (negative control) was pipetted, into a 96-well plate, and 200µl of reagent C was then added to each well. The plate was incubated in an orbital shaker at 37°C for 30 minutes, to allow the colour to develop. Then absorbance was measured at 562nm. Each sample was run in duplicate, with a 5% coefficient of variance accepted between duplicates. Samples which had a >5% coefficient of variance were repeated. A 10x or 20x multiplication factor was applied to all results to account for the appropriate dilution factor.

2.4.3 Western blot polyacrylamide gel electrophoresis procedure

A 12% gel was prepared using the reagents in the quantities detailed in Table 2.10. The gel was then poured into the casting unit using a syringe, with care taken to ensure no bubbles were introduced to the gel. Then, 5ml of water-saturated butanol was poured on top of the gel to prevent oxidation. After 90 minutes, the water-saturated butanol was washed off with distilled water, with any excess water being poured off and the gel blotted dry. The stacking gel was prepared to the specifications detailed in Table 2.11 and poured on top of the resolving gel, leaving a 1cm gap from the top of the chamber for a 0.75mm comb to be

inserted to form the wells, with care being taken that no bubbles were formed. The gel was left for 30 minutes to allow polymerisation.

Reagent	Quantity
Distilled water	9.9ml
30% polyacrylamide mix (37.5.1)	12ml
1.5M Tris (pH 8.8)	7.5ml
10% Sodium dodecylsulphate (SDS) (50g SDS/500ML water)	300µl
10% Ammonium persulphate (APS) (1g APS/10ml water)	300µl
Tetramethylethylenediamine (TEMED)	12µl

Table 2.10 Details and quantities of reagents used to prepare the resolving gel.

Reagent	Quantity
Distilled water	6.8ml
30% polyacrylamide mix (37.5.1)	1.7ml
1.5M Tris (pH 6.8)	1.25ml
10% Sodium dodecylsulphate (SDS) (50g SDS/500ML water)	100µl
10% Ammonium persulphate (APS) (1g APS/10ml water)	100µl
Tetramethylethylenediamine (TEMED)	10µl

Table 2.11 Details and quantities of reagents used to prepare the stacking gel.

2.4.3.1 Preparation of samples for electrophoresis

Proteins were diluted to 4.2µg/ml in 20µl using the calculation in Figure 2.9, in a sterile Eppendorf tube. Then, 50µl of protein dissociation buffer was added to each sample, followed by 14µl of glycerol-bromophenol blue loading dye. Samples were incubated in a water bath set at 100°C for 10 minutes in order to dissociate the proteins. When removed from the water bath the samples were immediately placed on ice.

$$\beta = \frac{[84 x \alpha / 10]/20}{x}$$

Figure 2.9 The equation used to calculate the amount of mitochondria required to produce a 4.2µg/l in 20µl protein concentration.

B = volume of stock mitochondria, α = desired protein content on gel, x = protein concentration mg/ml

2.4.3.2 Loading the gel and running electrophoresis

The combs were carefully removed from the gel, wells were rinsed with distilled water and then drained. The wells were then filled with 1X running buffer (5mM tris, 50mM glycine (Fisher Scientific), 2% SDS and distilled water) and 20µl of sample/15µl of protein molecular weight marker (Thermo Scientific) was loaded into the wells. Samples were loaded into the gel in a random order, which was carefully recorded. If any wells remained empty they were filled with dye to ensure even running. The electrophoresis chamber was then filled with 4.5 litres of 1X running buffer, and the gel chamber was carefully lowered in. The electrophoresis apparatus was connected to a power pack and the gel run at 150v (40mA) for 120 minutes or until the dye has progressed to 2cm away from the bottom of the gel.

2.4.3.3 Semi-dry protein transfer

Six pieces of Whatman blotting paper (Scientific Laboratory Supplies, UK) were cut to size and soaked in Towbin buffer (containing 25nM Tris, 192mM glycine, 20% v/v methanol, p.H 8.3) with approximately 0.09ml of Towbin buffer per $1cm^2$ of blotting paper. Three pieces of the blotting paper were then laid down on a Hoefer semi-dry transfer unit TE77X (Hoefer Inc. MA, USA), with care being taken to eliminate air bubbles. Then, 1 piece of nitrocellulose (GE Healthcare, UK) was added to the stack. Once the dye has progressed a suitable distance, the gel was removed from the electrophoresis chamber and placed onto the nitrocellulose membrane, with care being taken to ensure no air bubbles were formed. The remaining 3 pieces of Whatman paper were placed on top, forming a 'gel sandwich' (Figure 2.10). After assembly of the gel sandwich, the unit was run for 90 minutes at 0.8mA per $1cm^2$.

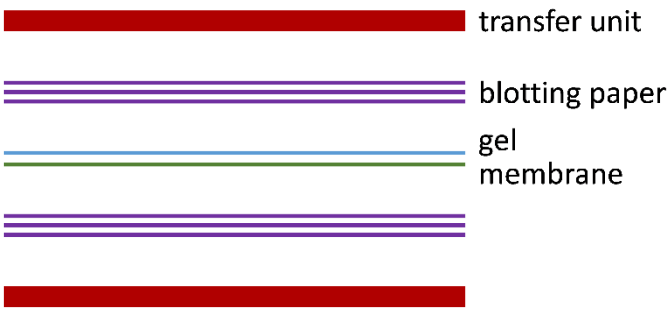

Figure 2.10 Illustration of the assembly of gel and membrane for semi-dry transfer.

2.4.3.4 Staining

After transfer the membrane was submerged in Tris buffered saline with Tween® 20 (TTBS), before being soaked in 100ml 1:10 Ponceau S red stain solution (containing 0.2g ponceau S, 3g trichloroacetic acid (Fisher Scientific), 3g sulphosalicylic acid (Acros Organics, NJ, USA) and distilled water) for 10 minutes. The excess stain was rinsed off with distilled water and the protein was visualised to ensure even loading. The positions of the molecular weight markers from the protein ladder were marked with an antigen/antibody pen (for rabbit primary antibodies, Alpha Diagnostic, TX, USA). The membrane was then rinsed in TTBS and placed in 50ml of blocking solution (10% w/v milk powder (Marvel, Premier Foods, Spalding, UK) in TTBS) and left overnight, at 4°C.

2.4.4 Western blot protein detection procedure

The membrane in the blocking solution was removed from the refrigerator and blocked at room temperature on a shaker for 30 minutes. The membrane was then removed from the blocking solution, rinsed with TTBS twice, then incubated in 15ml of the appropriate diluted primary UCP1 antibody, diluted to 1/10000 (antibody was raised in rabbit against sheep, developed and optimised in house[436]) for 1 hour at room temperature on a shaker. The membrane was then rinsed in 50ml of TTBS 3 times, for 10 minutes each time, at room temperature on a shaker. After this, the membrane was incubated for 60 minutes, on a shaker at room temperature, with 10ml of a swine anti-rabbit secondary antibody (P0217, Dako Ltd., Ely, UK) at a 1:20,000 dilution in a 3% (w/v) milk solution. Following this, the membrane was rinsed twice with TTBS and washed in in TTBS four times for 15 minutes each time, at room temperature on a shaker. Then, the membrane was rinsed in TBS four times and soaked in TBS for 30 minutes, at room temperature on a shaker and then soaked in TBS for a further 30 minutes at room temperature.

The membrane was placed into the solution of 9ml of hydrogen peroxide and 9ml of luminol reagent (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Watford, Hertfordshire, UK) and incubated at room temperature for 5 minutes. The membrane was subsequently removed from the solution and excess reagent was removed with blotting paper. The membrane was carefully placed and wrapped in cling film, avoiding the formation of air bubbles, then the membrane was imaged using a luminescent image analyser, with 1 minute exposure.

The densitometric evaluation was performed using Aida Image Analyzer v.4.15 (Raytek Scientific Ltd., Sheffield, UK). This evaluates the density of the bands, which reflects the abundance of polypeptide within the sample, and subtracts the intensity of the background staining.

2.5 Histology

Histology is the microscopic study of tissue, which enables researchers to visualise structure and cellular components. For histological analysis the tissue is fixed, in order to preserve it from degradation, and to maintain the cells structure. This works by cross-linking the proteins of a sample using a chemical fixative[437], such as formalin. After the tissue has been fixed, it undergoes processing. This takes part in 4 steps:

1) dehydration, where the tissue is immersed in a series of increasing concentrations of a dehydration agent, often ethanol. This is necessary due to paraffin wax being hydrophobic
2) clearing; immersion of the tissue in a clearing agent, such as xylene. This removes traces of the dehydration agent
3) infiltration; this is the immersion of the tissue sample in liquid wax
4) embedding; tissue is embedded in a liquid embedding material, such as paraffin wax, which is hardened, resulting in blocks containing the tissue, which can be sectioned. In this form, the tissue is relatively stable for storage

A microtome with a mounted blade is used to cut thin sections of tissue which a transferred onto a glass slide and dried, ready for further analysis.

2.5.1 Histological tissue processing procedure

Perirenal adipose tissue samples were fixed in a 10% formalin (10%v/v formaldehyde in 0.9% w/v sodium chloride/distilled water (Fisher scientific) saline solution) solution immediately upon dissection, and were stored at room temperature until processing.

A small amount of tissue (~1cm in length) was cut from each sample and transferred to a 30mm x 25mm x 5mm Histosette II cassette (Simport Scientific, QC, Canada). The cassettes were stored in 70% denatured ethanol awaiting processing, then processed in a Shandon Excelsior advanced tissue processor (Thermo Shandon Ltd, Runcorn, Cheshire, UK). The processor was set to a program which comprised of six 1 hour cycles of denatured ethanol at 20°C (which were graduated, with the first three being 75%, 90% and 95% denatured ethanol, and the final three being 100% denatured ethanol), three 1 hour cycles of xylene (Fisher Scientific) at 20°C and three 1 hour and 20 minute cycles of wax (Tissue-Tek Embedding Wax; Sakura Finetek, Alphen aan den Rijn, Netherlands) at 62°C. Upon

completion of the processing, each tissue sample was embedded in a paraffin wax block using a Tissue-Tek III Embedding Center (Sakura Finetek).

Sections for histological analysis were sliced at 5µm thickness from each block using a sledge microtome (AS200; Anglia Scientific, Cambridgeshire, UK). After slicing, sections were transferred into 50% denatured ethanol then floated in a water bath set to 45 °C. Each section was transferred onto a SuperFrost Plus glass microscope slide (Menzel-Gläser; Gerhard Menzel, Braunschweig, Germany) and placed onto a heat rack for 30 minutes to dry, then further dried in an oven set at 37 °C, for at least 48 hours.

2.5.2 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining is one of the most common staining techniques and makes use of two stains, Harris' haematoxylin and eosin. It allows the structure of a tissue to be visualised by staining the components.

In order to function as a stain, haematoxylin must first be oxidised to haematein then bound to one of several metal ions, including aluminium, iron and chromium. The metallic ion bound to a dye allows it to bind to tissue. Haematoxylin is positively charged, which means that it reacts with negatively charged cell components, such as nucleic acid, in the nucleus, to produce a blue stain. Haematoxylin is a regressive stain, meaning the stain is applied and the tissue overstained, then a differentiator is used to remove excess haematoxylin[438].

Eosin is negatively charged and acts as an acidic dye, which reacts with positively charged components of the tissue, such as amino groups present in proteins within the cytoplasm, which stain pink.

2.5.2.1 Haematoxylin and eosin staining procedure

One slide from each tissue sample was placed in a slide rack and dewaxed by immersion in two consecutive xylene troughs for 3 minutes, then rehydrated with two stages of 100% ethanol immersion and one stage of 70% ethanol/distilled water, finally being washed with distilled water. Slides were then stained by immersion in Harris's haematoxylin (VWR Ltd, Lutterworth, UK) for precisely 5 minutes, and then rinsed in a continual flow of fresh tap water for 5 minutes to remove excess stain. The haematoxylin stain was then regressed by immersion in acid-alcohol (1% 36N hydrochloric acid in 70% ethanol) solution for precisely 5 seconds, slides were then rinsed in tap water and "blued" off in alkaline Scott's tap water (a

0.2% sodium bicarbonate and 20% magnesium sulphate distilled water solution) for 1 minute. Slides were rinsed in tap water and immersed in a 1% Eosin Yellowish (VWR Ltd) counterstain for 3 minutes. Slides were then washed in running tap water for 1 minute 30 seconds to remove any excess staining. Following this, slides were dehydrated by two 2 minute immersions in 100% denatured ethanol, then ethanol cleared in two 3 minute xylene immersions. Sections were then mounted with glass coverslips (VWR Ltd), affixed using DPX mounting medium (Fisher Scientific), and left to dry overnight.

2.5.3 Perirenal adipocyte H&E analysis

H&E stained slides were scanned and visualised using a Leica DMRB microscope (Leica Microsystems, Milton Keynes, UK) and imaged with a Retiga-2000R CCD digital camera (QImaging, Surrey, BC, Canada) at 20X magnification.

The analysis was performed using Image-Pro Premier software (Media Cybernetics, Rockville, MD, USA). To calculate the mean volume of the white adipocytes, twenty 300x300µm boxes were randomly distributed over the image initially (Figure 2.11), after which, box placement was 'fine-tuned' by moving the box the minimum amount required to sufficiently avoid any areas of tissue with vessels, tears or large areas of brown adipocytes.

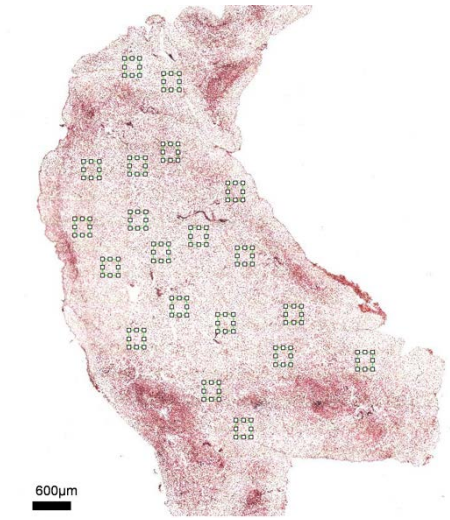

Figure 2.11 Illustrative image to illustrate the selection of areas for mean white adipocyte volume analysis.

Twenty 300x300µm boxes were evenly distributed across the tissue, then their position was amended so as not to cover any areas which were not adipocytes, such as vessels and tears.

Settings to detect white space on the tissue (i.e. the centre of white adipocytes) were suitably refined in order to ensure that the whole adipocyte was counted. The size of the white space which was to be counted was also refined, in order to ensure small 'white' areas, such as the small areas in a brown adipocyte, were not counted in the total area of white space.

This protocol was refined by myself, by trialling numerous numbers and sizes of boxes. The minimum and maximum white space detection limits were also refined manually by myself, by trialling different values and visually examining whether they sufficiently detected the areas which were white adipocytes.

The total area of white space for each 300x300µm area was measured by the software (Figure 2.12), and carefully recorded. Following this, each cell within each area was counted, using a 'tagging' tool, which placed a marker in each cell as it was clicked on, to avoid the same cell being counted twice. This tool also counted the number of tags, reducing the risk of the researcher making an error. The number of adipocytes per area was recorded, then adipocyte volume was calculated by dividing the total amount of white space for all areas, by the total number of adipocytes.

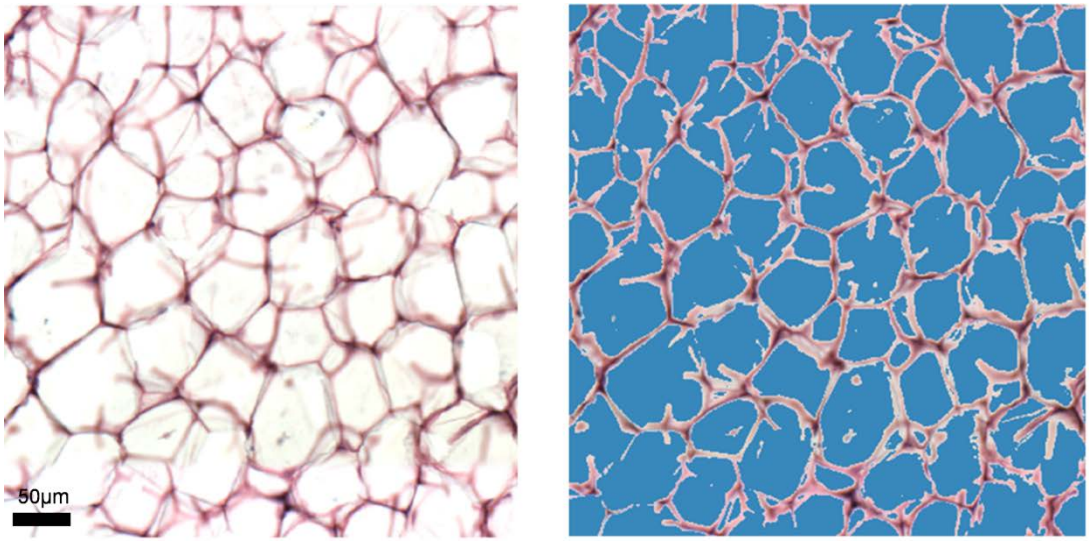

Figure 2.12 Illustrative image of white adipocyte volume analysis

The blue area represents the 'white' space which was counted. The researcher then manually counted the number of individual adipocytes, and the mean cell volume was calculated.

2.5.4 Immunohistochemistry

Immunohistochemistry (IHC) is a technique used for detecting antigens (proteins) of interest within a histological tissue sample. The principle is similar to that used in Western blotting, where the primary antibody binds to a protein of interest and the secondary antibody then binds to the primary antibody (See Figure 2.8, page 74). The enzyme HRP is commonly used as a label, in combination with 3,3'-Diaminobenzidine (DAB), which produces a brown colour when the target protein is detected.

When the tissue is counterstained, this allows the target protein and tissue morphology to be viewed together.

Before exposing to an antibody, it is important that slides are prepared correctly, by dewaxing and rehydration. This enables reagents to access the tissue. The fixation of the tissue in formalin results in its protein cross-linking. This is a problem, as it masks the antibody-binding sites (epitopes). In order to unmask the epitopes, and allow the antibodies to bind, a process known as heat-induced epitope retrieval is carried out, which usually involves heating with citrate buffer.

A positive control is a sample known to contain the target protein, in my studies, a 1 day old sample of ovine perirenal adipose tissue was used (Figure 2.13). A negative control is a sample of tissue, which undergoes the same treatment as the samples, however, is not exposed to the antibody (Figure 2.13).

2.5.4.1 Immunohistochemistry procedure

One slide per animal, plus one negative and one positive control were labelled with a random identification number and placed in a rack to be loaded into a Leica BondMax™ IHC slide processor (Leica Microsystems), and run on an automated software program (Vision Biosystems Bond version 3.4A, Leica Biosystems, Newcastle Upon Tyne, UK) using Bond polymer refine detection reagents (Leica Biosystems,).

This program first dewaxed the tissues with 3 applications of Bond Dewax Solution (an organic solvent containing > 98 % toluene based alkanes), followed by three rinses with 100% denatured ethanol. Then 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 room temperature. Samples were then treated with a Peroxide Block for 5 minutes at room temperature. All samples (except the negative control) were then incubated with 150µl of primary antibody against UCP1, which was diluted to 1 in 750 (optimised in house[401,436]) in Bond Primary Antibody Diluent (containing TBS, surfactant, protein stabiliser and 0.35% ProClin 950), for 15 minutes. Slides were then incubated for 8 minutes with 150µl of secondary antibody (HRP-conjugated secondary anti-mouse and rabbit antibody polymer). Slides were then incubated for 10 minutes in 150µl substrate Mixed DAB Refine (DAB Part 1 and DAB Part B in a ratio of 1:25 by volume).Following this, slides were rinsed 3 times with distilled water and counterstained, with 150µl of Harris's haematoxylin, for 5 minutes. Following each stage of the BondMax program, slides were washed with Bondwash Solution (supplied as a 10X concentrate containing TBS, surfactant and 3.5 % ProClin 950, pH 7.6) and distilled water. Sections were mounted with glass coverslips (VWR Ltd), affixed using DPX mounting medium (Fisher Scientific), and left to dry overnight.

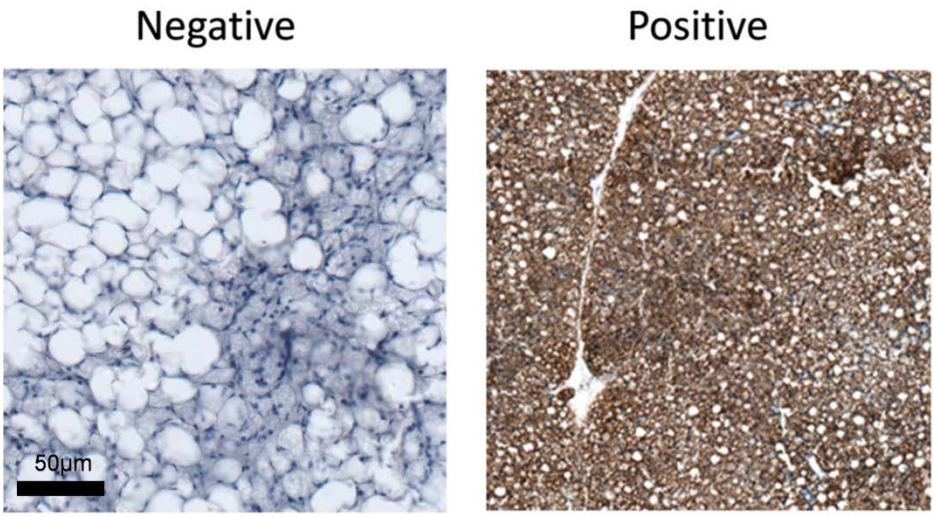

Figure 2.13 Example of negative and positive controls

One negative and one positive slide were included on each run on the BondMax. The negative slide was treated with all reagents except the antibody. The positive slide was processed in the same way as all other slides, and was a 1 day sample which was known to contain UCP1.

2.5.5 Immunohistochemistry quantification

The analysis of IHC was performed using Image-Pro Premier software (Media Cybernetics, Rockville, MD, USA). Images of the whole tissue section were cropped to remove the outer edge, as this is where the tissue was usually folded over, resulting in non-specific staining. The amount of 'brown' stain was quantified, by first measuring the total pigmented area (all colouration, including cell membranes), and secondly measuring the 'brown' colouration (positive UCP1 staining). Table 2.12 shows the values used to define the UCP1 stain colour, these values were manually refined by myself, in order to ensure that presence of the stain was not vastly over- or under-detected.

Colour	Integer
Red	80 – 240
Green	55 – 210
Blue	35 - 140

Table 2.12 Parameters used for the detection of the brown stain which indicates the presence of UCP1.

From this, a relative 'percentage' of brown staining was calculated. The unit of measurement is arbitrary, as whilst the amount of tissue on each slide was similar, they were not identical. This measure allowed me to compare treatment groups for relative UCP1 staining. Figure 2.14 illustrates how these values were applied and the software used to quantify the amount of UCP1 staining.

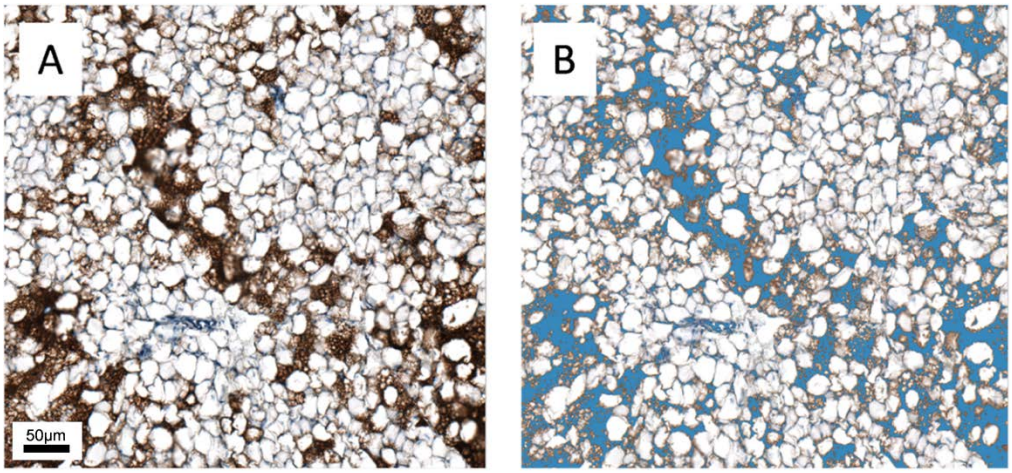

Figure 2.14 Illustrative image of quantifying UCP1 positive staining.

A) An example of an original UCP1 stained section, with the brown areas representing positive UCP1 staining. B) An example of the detection method, where the blue area represents the brown stained areas, which are quantified.

2.6 Plasma metabolite analysis

Plasma samples were thawed on ice and loaded into a Randox RX Imola auto-analyser (Randox Laboratories, County Antrim, UK). The samples were analysed in duplicate, and a calibration curve was completed for each metabolite analysed. All specific metabolite reagent kits were supplied by Randox. In the event of a sampling showing a variance coefficient >5%, analysis was repeated.

2.6.1 Acetate analysis

2.6.1.1 Summary

In living organisms, acetylation/deacetylation of proteins is vital for the posttranslational regulation of their functions. Acetyl-CoA is vital to the metabolism of carbohydrates and fats. Acetate has also been associated with the upregulation of UCP1 in rodents[439].

2.6.1.2 Principle

Acetate was measured using the principle enzymatic methods first described by Brown *et al*.[440]. Acetyl-CoA synthetase converts acetic acid to acetyl-CoA, which then reacts with oxaloacetate, in the catalysed by citrate synthase, resulting in citrate. Then, conversion of malate by L-malate dehydrogenase with the reduction of NAD to NADH leads to the formation of oxaloacetate. It is the NADH which is measured, at 340 nm.

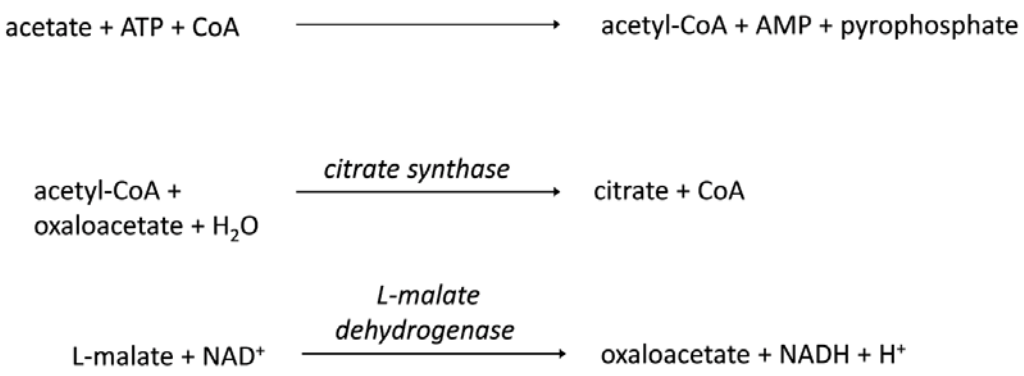

Figure 2.15 The enzymatic reaction involved in acetate analysis[440].

2.6.2 Total cholesterol analysis

2.6.2.1 Summary

Cholesterol is an essential structural component of all animal cell membranes, and so, is biosynthesised in all animal cells. It is transported in the plasma via lipoproteins.

2.6.2.2 Principle

The assay to detect total cholesterol is based on the enzymatic procedure first described by Flegg[441] in 1973, and the detection is based on Trinder's[442] colour system of peroxidase/phenol/4-aminoantipyrine.

Cholesterol is hydrolysed into free cholesterol and fatty acids by cholesterol esterase. The cholesterol is oxidised into cholestene-3-one and H2O2 in the reaction catalysed by cholesterol oxidase. The peroxidase catalyses the reaction between H2O2, 4-aminoantipyrine and hydroxybenzoate to produce a red compound. The colour intensity of this compound is proportional to the total cholesterol present in the sample.

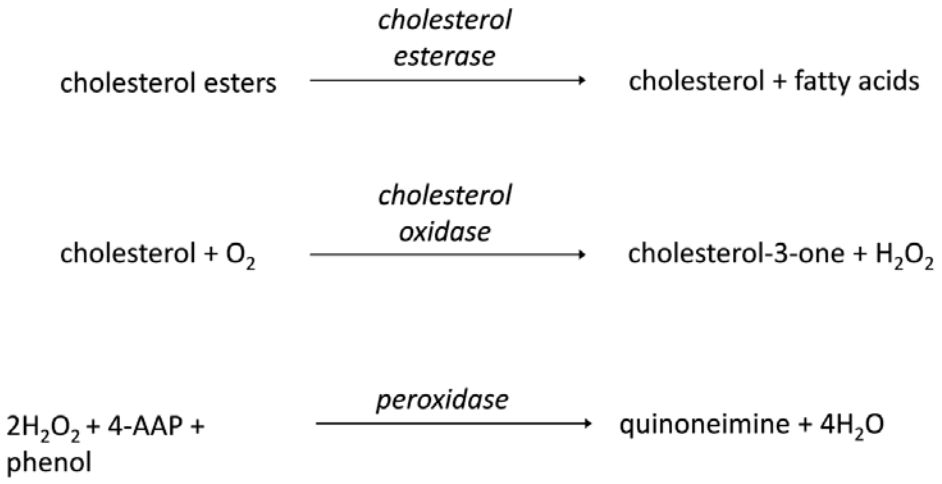

Figure 2.16 The enzymatic reaction involved in total cholesterol analysis[441-443].

2.6.3 Glucose analysis

2.6.3.1 Summary

Glucose present in the blood is the major source of cellular energy in the body. Dietary glucose is stored in the liver in the form of glycogen or converted to fatty acids and stored in the adipose tissues.

Elevated blood glucose levels, or hyperglycaemia, indicate diabetes mellitus and/or insulin resistance. Low levels of blood glucose, or hypoglycaemia, may be as a result of fasting or an inborn error of carbohydrate metabolism.

2.6.3.2 Principle

Glucose concentration can be determined using an adapted version of the enzymatic reaction described by Barham and Trinder[444], which uses Trinder's[445] colour system for detection, after enzymatic oxidation by glucose oxidase, that forms hydrogen peroxide, which then reacts under the catalysis of peroxidase with phenol and 4- aminophenazone, forming a red/violet quinoeimine dye. The absorbance can be measured at 500mn, Figure 2.17.

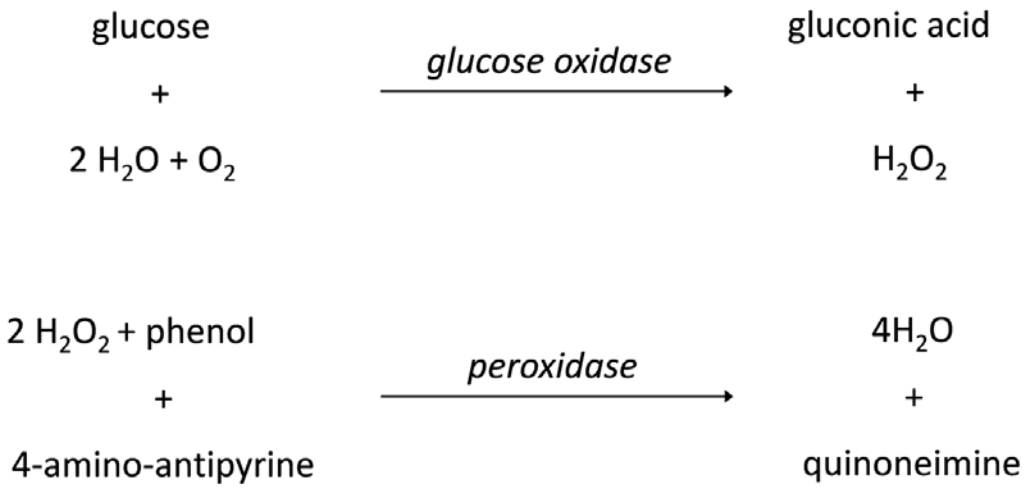

Figure 2.17 The enzymatic reaction involved in glucose analysis[444,445].

2.6.4 NEFAs analysis

2.6.4.1 Summary

NEFAs are fatty acids released from triglycerides and transported in the blood. Upon fasting, NEFAs are released from triglyceride storage and used as energy. Elevated NEFAs can indicate diabetes mellitus and insulin resistance.

2.6.4.2 Principle

Plasma NEFA concentration is measured by reaction with acyl-coenzyme-A synthetase, which, in the presence of ATP and coenzyme-A forms complex thiol esters of coenzyme-A and hydrogen peroxide[446], along with the by-products adenosine monophosphate and pyro-phosphate. This hydrogen peroxide is then oxidised by peroxidase with N-ethylN(2hydroxy-3-sulphopropyl)m-toluidine and 4-aminoantipyrine, and forms a purple coloured end product[447], with an absorbance at 550 nm, shown in Figure 2.18.

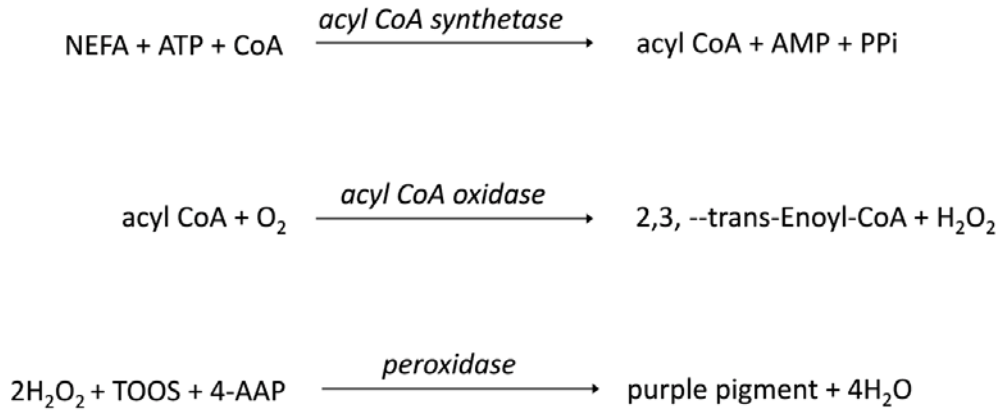

Figure 2.18 The enzymatic reaction involved in non-esterified fatty acid analysis[446,447].

2.6.5 Triglycerides analysis

Triglycerides are the main form in which fatty acids are stored in the body. NEFAs can be synthesised into triglycerides for storage, then when required, triglycerides are hydrolysed to NEFAs which enter the bloodstream. Elevated triglycerides are implicated in atherosclerosis, heart disease and stroke as well as in pancreatitis.

2.6.5.1 Summary

Triglycerides can be measured by the hydrolysis of triglyceride by lipase enzymes, which break down triglycerides into glycerol and free fatty acids. The glycerol which is released is phosphorylated to glycerol-3-phosphate, in a reaction catalysed by glycerol kinase, which is then oxidised by glycerol phosphate oxidase to dihydroxyacetone and hydrogen peroxide. The hydrogen peroxide undergoes a redox-coupled reaction with 4-aminoantipyrine, catalysed by peroxidase, which produces a purple coloured end product.

2.6.5.2 Principle

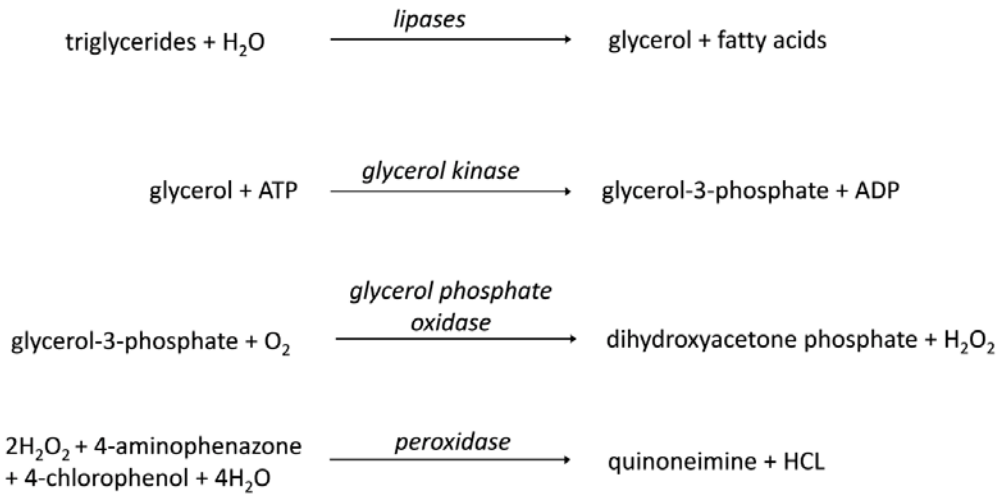

Figure 2.19 The enzymatic reaction involved in triglyceride analysis[448-450].

2.6.6 Urea analysis

2.6.6.1 Summary

Urea is synthesised in the liver from ammonia, as a result of deamination of amino acids, and therefore, the end product of protein metabolism. This is the body's primary means of the excretion of surplus nitrogen. Increased levels are found in renal diseases, urinary obstructions, shock and congestive heart failure. Decreased levels are found in liver failure and pregnancy.

2.6.6.2 Principle

Urea is hydrolysed to ammonia and carbon dioxide, by the enzyme urease. The ammonia combines with α-Ketoglutarate and reduced nicotinamide adenine dinucleotide (NADH), forming glutamate and nicotinamide adenine dinucleotide. The oxidation rate of NADH to NAD is measured as a decrease in absorbance in a certain time period. This is proportional to the urea concentration in the sample (Figure 2.20).

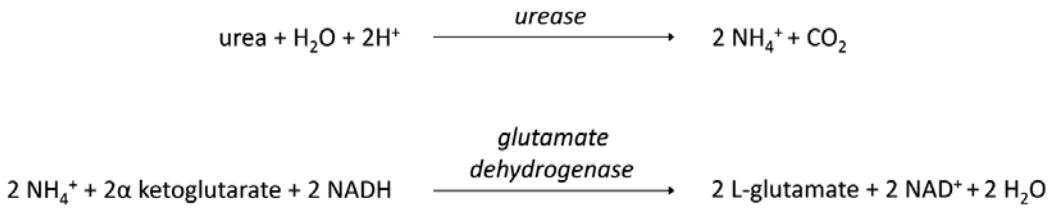

Figure 2.20 the enzymatic reaction involved in urea analysis[451].

2.7 Statistical analysis

Statistical analysis was carried out and graphs were created using GraphPad Prism v6.0 (GraphPad Software, CA, USA). Data is presented as mean ± standard error of the mean (SEM).

Parametric tests were used as non-parametric tests are not suitable for the analysis of small data sets[452]. For Chapter 3 (Study A) unpaired t-tests were used to compare control and canola groups. For chapters 4 and 5 (Study B) unpaired t-tests were used to test significance between control vs. canola and control vs. sunflower. The underlying purpose of the study was to examine the effect of supplementation, so there was no real benefit of directly comparing the results of the two supplemented groups.

Adjustments were not made for multiple comparisons (e.g. Bonferroni). This is due to the belief that such tests are overly rigorous, and whilst they have the benefit of reducing the number of type 1 errors (false positives), they increase the risk of type 2 errors (false negatives). A paper by Perneger[453] eloquently describes how the Bonferroni correction has limited applications in biomedical research and should not be used when assessing evidence with a specific hypothesis.

In Chapter 6, data was non-parametric and significance was tested using the Kruskal-Wallis one way analysis of variance for non-parametric data, with the Dunn's correction applied to correct for multiple group testing.

Results were considered to be significant when $p<0.05$.

No power calculations were carried out prior to my investigations, however, *post hoc* calculations were carried out where necessary using PS: Power and Sample Size Calculation version 3.1.2, 2014.

3 The effects of supplementing ewe diet with fatty acids during lactation on milk fatty acid profile and offspring adipose tissue thermogenic capacity

3.1 Introduction

This study (Study A) was carried out to assess the feasibility of a dietary intervention study, supplementing the diet of ewes during lactation, involving milk sampling and the sampling of offspring's adipose tissue. I analysed the samples which were obtained in this study.

As mentioned in Chapter 1, BAT has the propensity to utilise stored energy in order to produce heat[76], a process mediated by *UCP1*[76]. The discovery of 'brite' adipose tissue has also attracted attention in recent years, as potentially inducible 'brown-like' thermogenic adipocytes[454]. There is research *in vitro* and in rodents to suggest that CLA may play a role in activating *UCP1*[330,333,334], although the exact mechanisms by which this occurs are unknown.

Other studies have found that supplementing lactating ruminants with canola oil can induce effects on the milk fatty acid profile including increased MUFAs, decreased SFAs and an increase in CLA, in particular, the *cis*-9, *trans*-11 isomer[406,407,410]. These studies were conducted in goats and cows and, at present, to my knowledge no similar investigations have been carried out in sheep.

In my investigation, the milk fatty acid profile was investigated and then mRNA expression of *UCP1* and other genes associated with thermogenesis was assessed in offspring's perirenal adipose tissue, along with expression of genes associated with WAT and brite adipose tissue.

3.2 Hypotheses

For this study, reflecting the results of studies in goats and cows described above, I hypothesised that, in comparison to controls, the milk of canola supplemented ewes would be:

- lower in SFAs.
- higher in MUFAs.
- higher in total CLA and *cis*-9, *trans*-11 CLA.

I then hypothesised that, due to elevated milk CLA concentrations, the lambs of supplemented ewes would:

- have increased UCP1 and expression of related thermogenic genes.
- show a difference in adipose tissue morphology, with the presence of more multilocular brown adipocytes.

3.3 Methods

A detailed description of all the methods used in this chapter can be found in Chapter 2. The experimental model used in this chapter is summarised in Figure 3.1. All animal experimentations for this study were performed by Professor Michael Symonds, Dr Mark Birtwistle, Dr Stuart Astbury and Dr Viv Perry at the University of Nottingham, Sutton Bonington campus. The sheep milk samples were sent to The University of Pecs, Hungary to be analysed by Dr Tamás Marosvölgyi. A full description of techniques used for gene expression, histological and protein analyses are detailed in Chapter 2, (Section 2.5) and a description of statistical analysis performed (Section 2.7, Page 94).

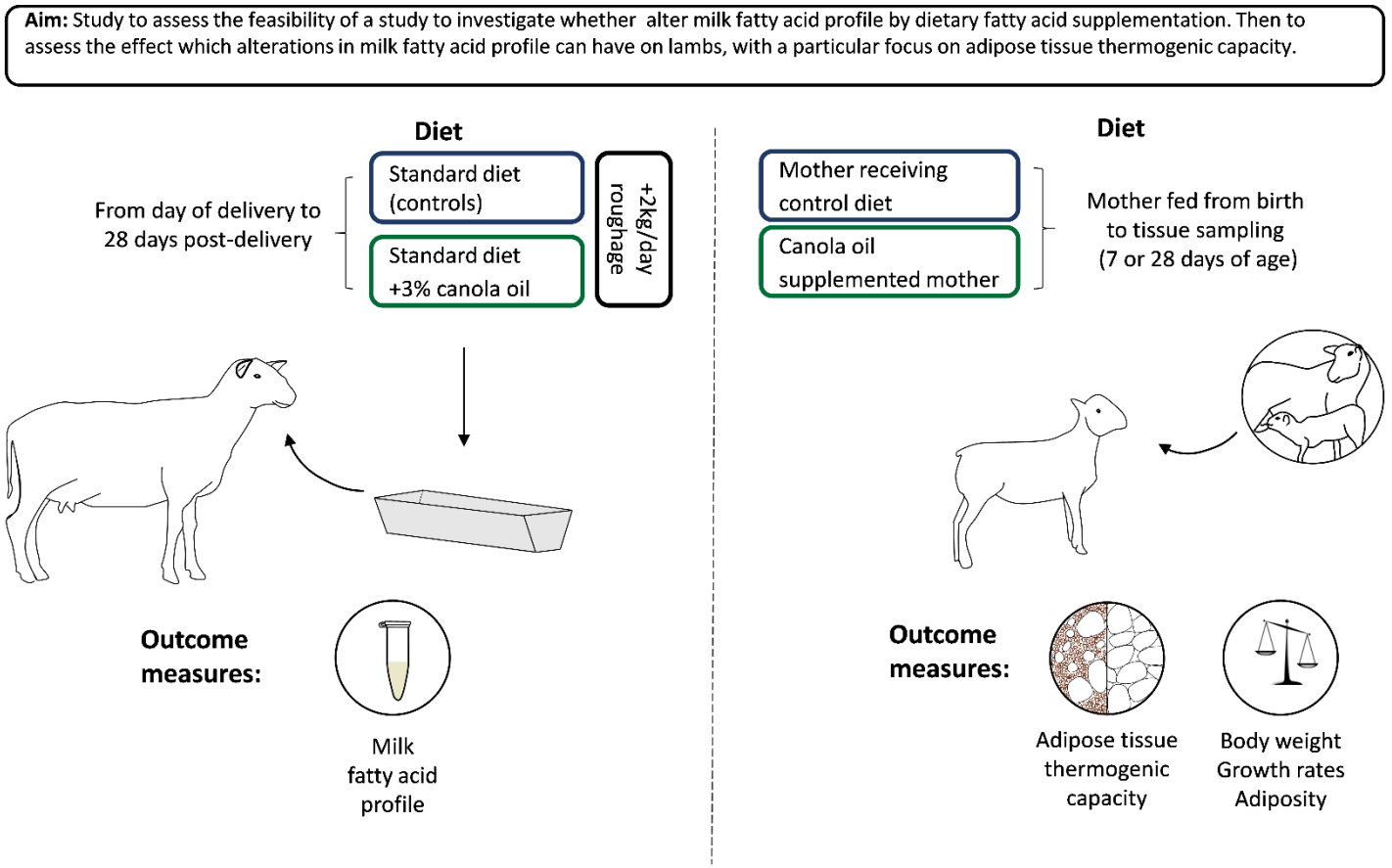

Figure 3.1 Summary of experimental design.

The day numbers refer to days postpartum for ewes and days of age for lambs.

3.4 Results

3.4.1 Effects of maternal canola oil supplementation on milk fatty acid profile

Milk was obtained from all 10 ewes in the canola group at 7 days postpartum, and 9 of the ewes in the control group (milk was not obtained from one of the control ewes, as attempted and was abandoned to avoid causing undue stress to the animal). At 28 days, milk was obtained from all 10 canola group ewes, and 9 control group ewes. Analysis of all milk samples was not possible, due to macroscopic contamination particles being present within the samples. Number of samples is given for each analysis in the caption of every graph.

3.4.1.1 Total fat

Supplementation of canola oil had no significant effect on the total fat content of milk (w/w%) at either 7 or 28 days of lactation, as shown in Figure 3.2.

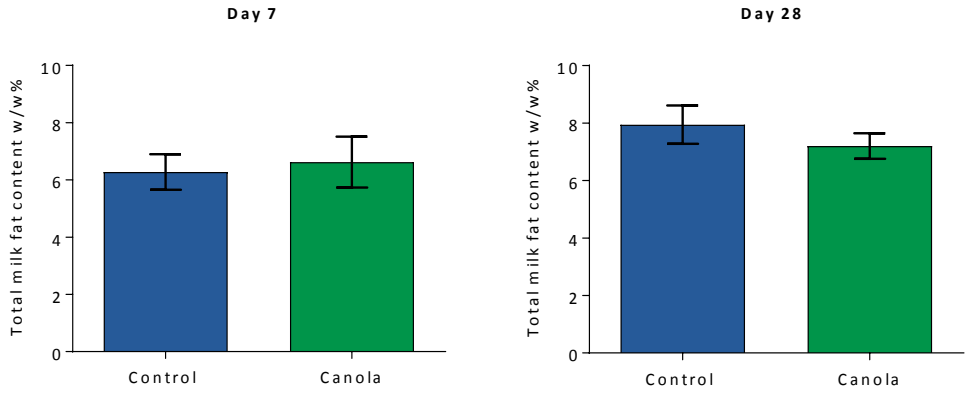

Figure 3.2 Milk fat content (w/w%) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control; day 7 n=6, day 28 n=9), or a standard diet + 3% canola oil (canola; day 7 n=9, day 28 n=10). w/w% = percentage by weight of total solution. Values are mean ± SEM.

3.4.1.2 Saturated fatty acids

Maternal supplementation of canola oil from the onset of lactation had very little effect on the SFA content of milk sampled at 7 or 28 days. There were no significant changes at 7 days between ewes supplemented with 3% canola oil and controls. At 28 days, out of the 10 SFAs tested, two showed modest changes. Palmitic acid (C16:0) levels in the milk of canola supplemented ewes were 9% lower than controls at 28 days ($p=0.018$). Stearic acid (C18:0) levels showed a 17% increase in milk sampled from canola supplemented ewes at 28 days ($p=0.018$). No changes were observed in any other SFAs tested, all data is shown in Table 3.1.

Fatty acid	Common name	Day 7			Day 28		
		Control (n=6)	Canola (n=9)	Effect (% change)	Control (n=9)	Canola (n=10)	Effect (% change)
C8:0	Caprylic Acid	1.43±0.1	1.68±0.1	p=0.20	2.22±0.2	2.16±0.2	p=0.81
C10:0	Capric Acid	2.81±0.2	3.47±0.3	p=0.12	5.50±0.6	4.95±0.5	p=0.49
C11:0	Undecylic Acid	0.0083±0.005	0.0033±0.002	p=0.32	0.024±0.01	0.009±0.004	p=0.22
C12:0	Lauric Acid	1.55±0.1	1.80±0.1	p=0.17	2.66±0.3	2.37±0.2	p=0.39
C13:0	Tridecylic Acid	0.028±0.01	0.029±0.01	p=0.95	0.05±0.01	0.048±0.004	p=0.77
C14:0	Myristic Acid	4.56±0.2	4.92±0.2	p=0.33	6.88±0.3	6.27±0.2	p=0.15
C16:0	Palmitic Acid	20.60±0.7	19.86±0.5	p=0.39	23.62±0.5	21.60±0.6*	9% ↓p=0.018
C18:0	Stearic Acid	18.79±0.4	19.52±0.6	p=0.26	14.65±0.79	17.13±0.6*	17%↑p=0.018
C20:0	Arachidic Acid	0.42±0.04	0.034±0.05	p=0.11	0.33±0.04	0.34±0.05	p=0.98
C22:0	Behenic Acid	0.073±0.005	0.086±0.006	p=0.17	0.11±0.009	0.12±0.009	p=0.33

Table 3.1 Milk saturated fatty acid content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control) or a standard diet + 3% canola oil (canola). Values are mean ± SEM. Within sampling day, the control and canola group were compared by an unpaired t-test. Significant differences are denoted by directional arrows.

3.4.1.3 Monounsaturated fatty acids

There were no effects of maternal canola supplementation on milk MUFA content at either 7 or 28 days, shown in Figure 3.3.

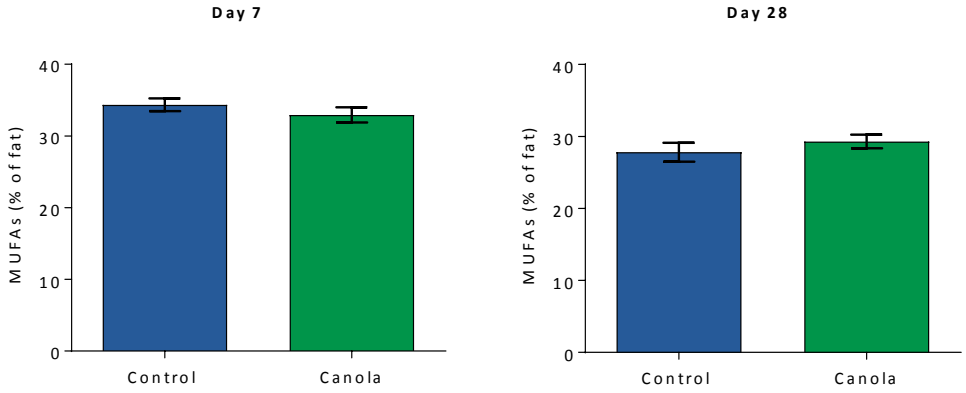

Figure 3.3 Milk monounsaturated fatty acid (MUFA) content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control; day 7 n=6, day 28 n=9), or a standard diet + 3% canola oil (canola; day 7 n=9, day 28 n=10). Values are mean ± SEM.

No effects were seen in oleic acid (C18:1 n-9) at either time point (Table 3.2). There was no effect of supplementation on erucic acid at 7 days, but at 28 days ewes supplemented with canola oil has 52% more erucic acid present in the milk (p=0.0039). Nervonic acid was elevated at 7 days in the milk of canola oil supplemented ewes (33%, p=0.0073), but not 28 days. All data is shown in Table 3.2.

		Day 7			Day 28		
Fatty acid	**Common name**	**Control (n=6)**	**Canola (n=9)**	**Effect of supplement (% change)**	**Control (n=9)**	**Canola (n=10)**	**Effect of supplement (% change)**
C18:1 n-9	**oleic acid**	33.65±0.9	32.21±1	p=0.35	27.06±1.3	28.57±0.9	p=0.36
C22:1 n-9	**erucic acid**	0.0095±0.0003	0.0180±0.004	p=0.15	0.0142±0.001	0.0216±0.002	52% ↑P=0.0039
C24:1 n-9	**nervonic acid**	0.015±0.0009	0.02±0.001	33% ↑P=0.0073	0.025±0.003	0.031±0.002	p=0.11

Table 3.2 Milk omega-9 monounsaturated fatty acid content (% of fat) at 7 and 28 days of lactation.

Samples from ewes fed a standard diet (control) or a standard diet + 3% canola oil (canola). Values are mean ± SEM. Within sampling day, the control and canola group were compared by an unpaired t-test. Significant differences are denoted by directional arrows.

3.4.1.4 Omega-3 and -6 fatty acids

Results for milk omega-3 and -6 fatty acid content are shown in Table 3.3.

At 7 days, supplementation had no effect on any of the omega-3 or 6 fatty acids analysed.

At 28 days, there were no effects of supplementation on any of the omega-3 fatty acids, but there was a 13% reduction in total omega-6 PUFAs in the milk of ewes supplemented with canola oil ($p=0.029$). There was also a similar, 14%, reduction in linoleic acid (LA, C18:2 n-6, $p=0.022$). There were no other significant changes in any of the other omega-6 fatty acids analysed.

			Day 7			Day 28		
	Fatty acid	Common name	Control (n=6)	Canola (n=9)	Effect (% change)	Control (n=9)	Canola (n=10)	Effect (% change)
Omega-3	n-3 PUFA	Omega-3 PUFAs	1.40±0.07	1.33±0.1	p=0.64	1.38±0.05	1.29±0.07	p=0.37
	n-3 LCPUFA	Omega-3 LCPUFAs	0.37±0.03	0.42±0.03	p=0.26	0.45±0.03	0.46±0.03	p=0.83
	C18:3 n-3	α-Linolenic acid (ALA)	1.02±0.05	0.90±0.1	p=0.41	0.92±0.05	0.83±0.05	p=0.23
	C18:4 n-3	Stearidonic acid	0.0099±0.0007	0.0120±0.001	p=0.21	0.011±0.0007	0.0097±0.001	p=0.55
	C20:3 n-3	Eicosatrienoic acid	0.016±0.002	0.018±0.0006	p=0.46	0.017±0.0009	0.015±0.0008	p=0.12
	C20:5 n-3	Eicosapentaenoic acid (EPA)	0.12±0.007	0.13±0.009	p=0.41	0.12±0.005	0.12±0.006	p=0.94
	C22:5 n-3	Docosapentaenoic acid	0.16±0.01	0.19±0.02	p=0.25	0.23±0.02	0.24±0.02	p=0.75
	C22:6 n-3	Docosahexenoic acid (DHA)	0.073±0.01	0.088±0.01	p=0.36	0.089±0.01	0.093±0.006	p=0.80

Continued overleaf

	Fatty acid	Common name	Day 7			Day 28		
			Control (n=6)	Canola (n=9)	Effect (% change)	Control (n=9)	Canola (n=10)	Effect (% change)
Omega-6	n-6 PUFA	Omega-6 PUFAs	2.18±0.1	2.07±0.05	p=0.35	2.42±0.1	2.11±0.05	13%↓p=0.029
	n-6 LCPUFA	Omega-6 LCPUFAs	0.20±0.01	0.21±0.009	p=0.31	0.24±0.01	0.23±0.005	p=0.72
	C18:2 n-6	Linoleic acid (LA)	2.01±0.1	1.89±0.048	p=0.26	2.23±0.1	1.92±0.05	14%↓p=0.022
	C18:3 n-6	γ-linolenic acid	0.0078±0.002	0.0086±0.002	p=0.78	0.0092±0.002	0.0098±0.002	p=0.78
	C20:2 n-6	Eicosadienoic acid	0.032±0.002	0.036±0.002	p=0.23	0.037±0.001	0.038±0.002	p=0.76
	C20:3 n-6	Dihomo- γ-linolenic acid	0.016±0.001	0.017±0.001	p=0.74	0.020±0.001	0.021±0.001	p=0.71
	C20:4 n-6	arachidonic acid (AA)	0.10±0.007	0.11±0.005	p=0.78	0.11±0.006	0.11±0.003	p=0.66
	C22:2 n-6	Docosadienoic acid	NA	NA	NA	0.0024±0.001	0.0025±0.0007	p=0.98
	C22:4 n-6	Adrenic acid	0.0076±0.002	0.011±0.003	p=0.45	0.015±0.001	0.014±0.003	p=0.63

Table 3.3 Milk omega-3 and 6 fatty acid content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control) or a standard diet + 3% canola oil (canola). Values are mean ± SEM. Within sampling day, the control and canola group were compared by an unpaired t-test. Significant differences are denoted by directional arrows. PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid.

3.4.1.5 *Trans* fatty acids

There was no effect of maternal canola oil supplementation on the TFA content of the milk at either 7 or 28 days, as shown in Figure 3.4.

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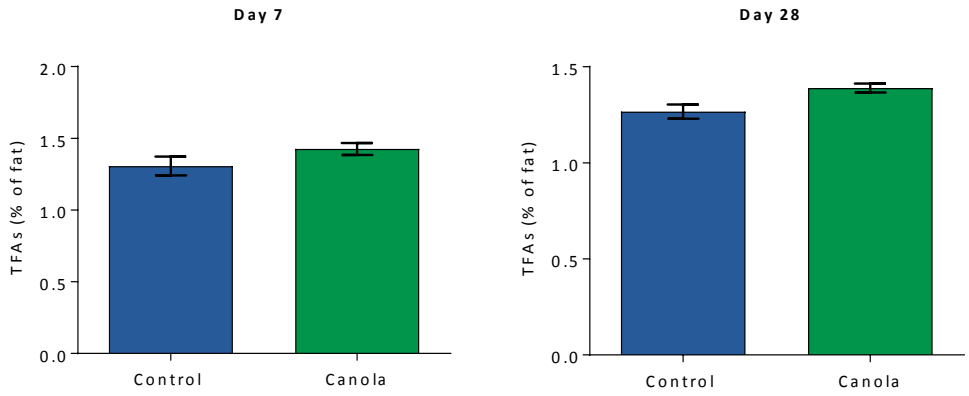

Figure 3.4 Milk trans fatty acid (TFA) content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control; day 7 n=6, day 28 n=9), or a standard diet + 3% canola oil (canola; day 7 n=9, day 28 n=10). Values are mean ± SEM.

3.4.1.6 Total CLA

Total milk CLA content was not affected by maternal canola oil supplementation at either 7 or 28 days of lactation (Figure 3.5).

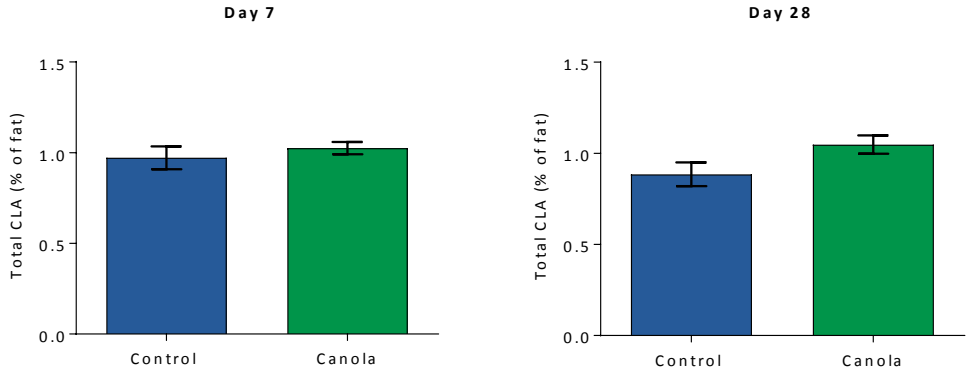

Figure 3.5 Milk total conjugated linoleic acid (CLA) content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control; day 7 n=9, day 28 n=9), or a standard diet + 3% canola oil (canola; day 7 n=9, day 28 n=9). Values are mean ± SEM.

3.4.1.7 *Cis*-9, *trans*-11 CLA isomer

Milk content of the *cis*-9, *trans*-11 CLA isomer was not affected by maternal canola oil supplementation at either the 7 or 28 day time point (Figure 3.6).

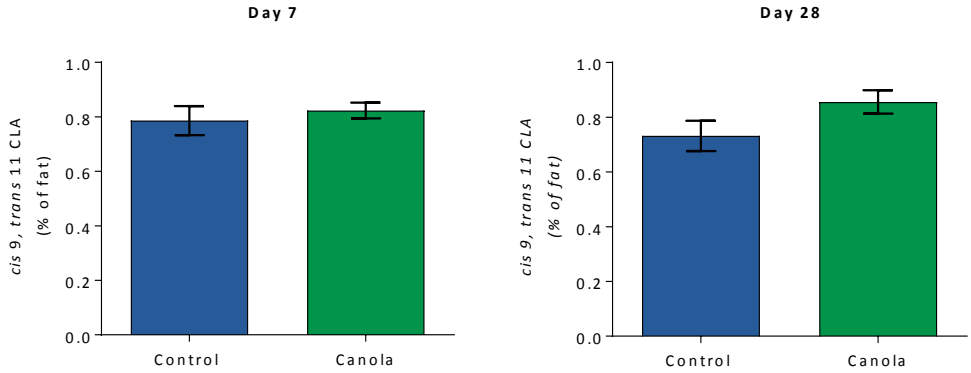

Figure 3.6 Milk *cis*-9, *trans*-11 conjugated linoleic acid (CLA) content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control; day 7 n=9, day 28 n=9), or a standard diet + 3% canola oil (canola; day 7 n=9, day 28 n=9). Values are mean ± SEM.

3.4.1.8 *Trans*-10, *cis*-12 CLA isomer

Milk *trans*-10, *cis*-12 CLA was unaffected by maternal canola oil supplementation, at both 7 and 28 days of lactation, as shown in Figure 3.7. An increase in the mean *trans*-10, *cis*-12 CLA content of the canola group's milk at day 28 is not statistically significant (p=0.2).

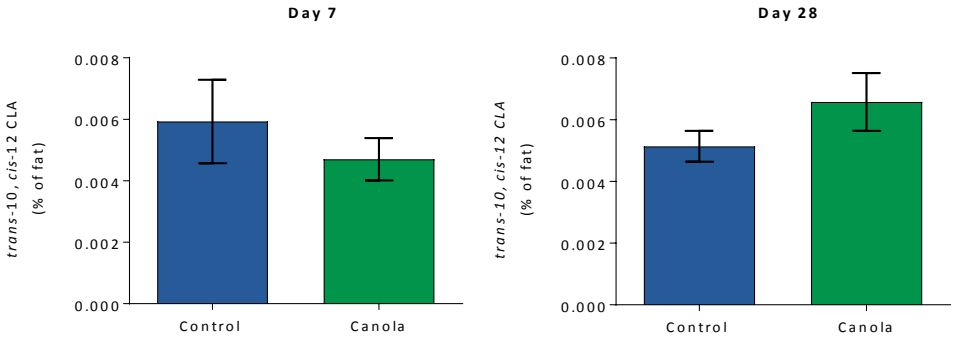

Figure 3.7 Milk *trans*-10, *cis*-12 conjugated linoleic acid (CLA) content (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control; day 7 n=9, day 28 n=9), or a standard diet + 3% canola oil (canola; day 7 n=9, day 28 n=9). Values are mean ± SEM.

3.4.2 Other CLA isomers

Aside from the two main biologically active isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12), ten other CLA isomers were tested. Overall, there were few significant changes in the milk content of these CLA isomers between the control and canola group. One exception was the *trans*-7, *cis*-9 isomer, which was 24% (p=0.009) and 73% (p<0.0001) higher in the canola group's milk at 7 and 28 days respectively. No changes were observed in any other isomers at 7 days, but at 28 days, the *trans*-11, *trans*-13 isomer was raised by 29% in the canola group (p=0.03) and the *trans*-10, *trans*-12 isomer was 54% higher in the canola group (p=0.001). All data is shown in Table 3.4.

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Isomer	Day 7			Day 28		
	Control (n=9)	Canola (n=9)	Effect of supplement (% change)	Control (n=9)	Canola (n=9)	Effect of supplement (% change)
***trans*-7, *cis*-9**	0.029±0.002	0.036±0.002	24%↑p=0.0090	0.033±0.002	0.057±0.003****	73%↑ p<0.0001
***trans*-12, *trans*-14**	0.016±0.001	0.019±0.001	p=0.13	0.011±0.001	0.014±0.001	p=0.15
***trans*-11, *trans*-13**	0.029±0.002	0.032±0.002	p=0.21	0.021±0.002	0.027±0.002*	29%↑p=0.03
***trans*-10, *trans*-12**	0.003±0.0002	0.0034±0.0002	p=0.26	0.0037±0.0002	0.0057±0.0004***	54%↑p=0.001
***tra*ns-9, *trans*-11**	0.016±0.0007	0.016±0.0008	p=0.91	0.017±0.001	0.019±0.001	p=0.24
***trans*-8, *trans*-10**	0.0031±0.0002	0.0034±0.0002	p=0.33	0.0042±0.0002	0.0041±0.0002	p=0.69
***trans*-7, *trans*-9**	0.0029±0.0001	0.0032±0.0002	p=0.18	0.003411±0.0002	0.0038±0.0001	p=0.12
***cis*-12, *trans*-14**	0.0067±0.0005	0.0064±0.0004	p=0.62	0.0050±0.0005	0.0043±0.0003	p=0.25
***trans*-11, *cis*-13**	0.065±0.007	0.068±0.004	p=0.66	0.039±0.006	0.040±0.004	p=0.90
***trans*-8, *cis*-10**	0.0099±0.007	0.011±0.0005	p=0.32	0.011±0.0006	0.011±0.0004	p=0.32

Table 3.4 Milk content of other conjugated linoleic acid (CLA) isomers (% of fat) at 7 and 28 days of lactation.

Milk samples taken from ewes fed a standard diet (control) or a standard diet + 3% canola oil (canola). Values are mean ± SEM. Within sampling day, the control and canola group were compared by an unpaired t-test. Significant differences are denoted by directional arrows.

3.4.3 Lamb body weights

Body weights were not recorded for all lambs at all time points due to technical difficulties. The number of lambs for each group is detailed in the figure captions.

At birth, there were no significant differences in the weights of lambs of either gender regardless of dietary group. At 7 days of age and 28 days of age, there were no significant differences in the mean weights of lambs by maternal dietary group, in either gender illustrated in Figure 3.8.

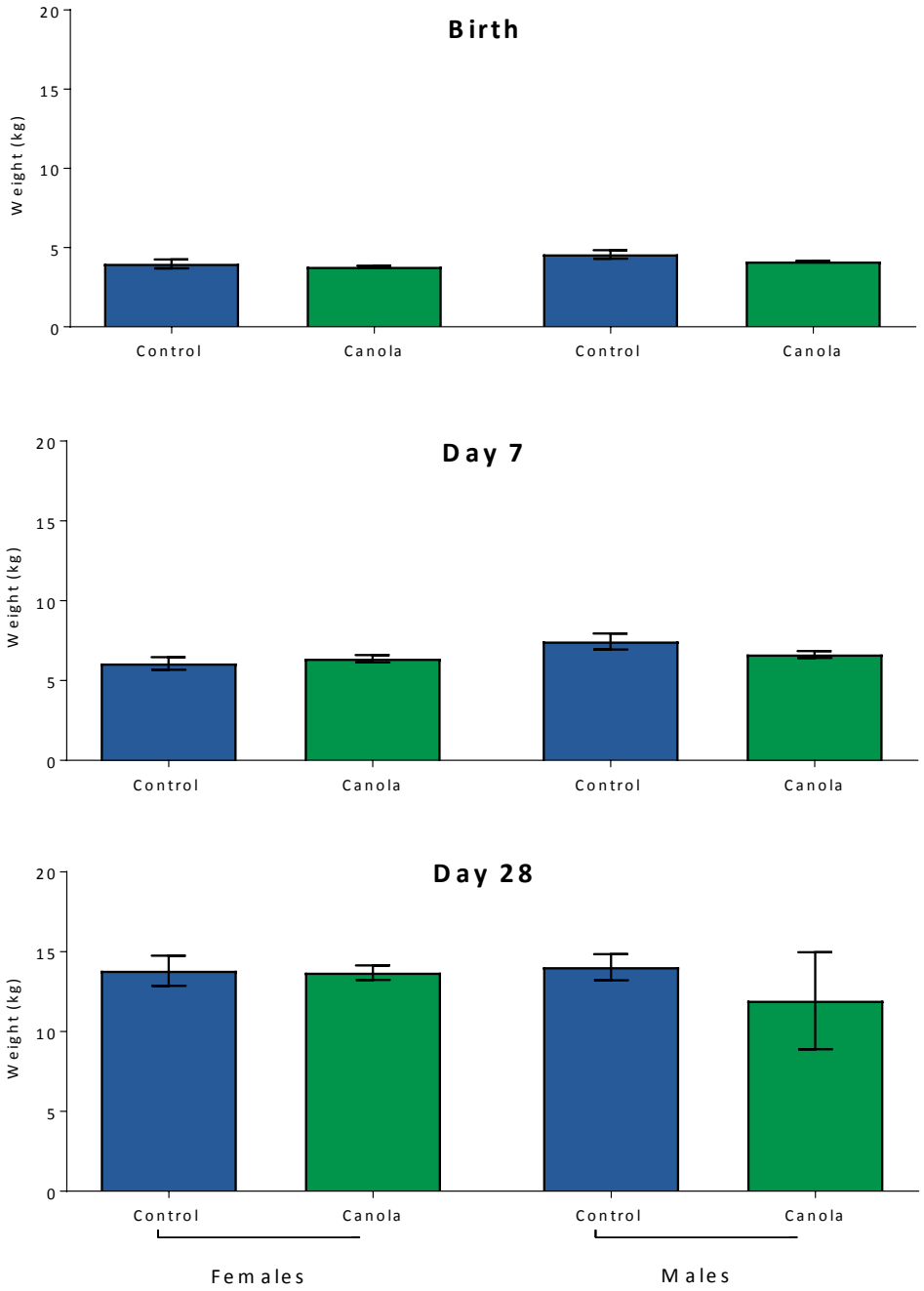

Figure 3.8 Lamb body weights (kg), by dietary group and gender, at birth, 7 and 28 days.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Birth; females (control, n=9; canola, n=11), males (control, n=6; canola, n=5). Day 7: females (control, n=9; canola, n=12), males (n=6 per group). Day 28: females (n=7 per group), males (n=3 per group). Values are mean ± SEM. Control and canola groups were compared by an unpaired t-test for each gender. No significant results were found (p≥0.05).

Growth rates between birth and day 7 did not differ significantly between genders or dietary groups (Figure 3.9).

Unfortunately, due to low numbers of animals with both 7 and 28 day weights recorded, it was not possible to make meaningful comparisons of their growth rates between these time points.

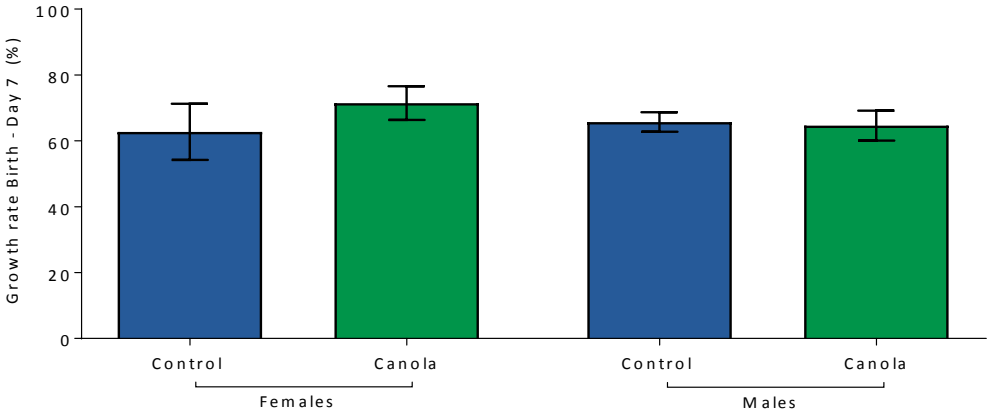

Figure 3.9 Lamb growth rates (percentage increase in weight), by dietary group and gender from birth to 7 days.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Females (controls, n=7; canola, n=10). Males (n=5 per group). Values are mean ± SEM. Control and canola groups were compared by an unpaired t-test for each gender. No significant results were found (p≥0.05).

3.4.4 Lamb organ weights

There were no differences in relative organ weights at day 7, between dietary groups in either gender (Table 3.5). At day 28 the relative liver weights of female lambs in the canola group were 9% lower than control females (p=0.046). This effect was not observed in males and there were no other effects of the dietary intervention on organ weights. Data is shown in Table 3.6.

	Females			Males		
	Control (n=6)	Canola (n=6)	p value	Control (n=4)	Canola (n=4)	p value
Adrenal	0.10±0.01	0.11±0.01	p=0.68	0.10±0.02	0.12±0.01	p=0.37
Brain	8.51±0.8	7.84±0.3	p=0.45	6.32±0.5	7.03±0.4	p=0.30
Heart	7.30±0.3	5.81±0.8	p=0.12	7.44±0.5	6.59±0.3	p=0.20
Kidneys	5.91±0.2	5.80±0.2	p=0.70	6.22±0.4	6.01±0.1	p=0.62
Liver	26.34±1.2	25.57±0.4	p=0.57	28.66±0.4	27.98±1.8	p=0.73
Lungs	21.27±1.4	20.40±0.6	p=0.57	20.97±2.0	19.95±0.9	p=0.66
Pancreas	0.82±0.1	0.99±0.2	p=0.50	0.73±0.2	0.87±0.2	p=0.55
Spleen	3.23±0.2	3.00±0.2	p=0.44	2.92±0.3	2.80±0.1	p=0.73
Thymus	1.50±0.2	1.18±0.2	p=0.26	0.81±0.1	0.97±0.3	p=0.63
Thyroid	0.16±0.04	0.12±0.02	p=0.33	0.09±0.003	0.12±0.02	p=0.10

Table 3.5 Lamb organ weights, relative to body weight (g/kg) at 7 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Values are mean ± SEM. Control and canola groups were compared by an unpaired t-test for each gender.

	Females			Males		
	Control (n=7)	Canola (n=7)	**Effect (% change)**	Control (n=3)	Canola (n=3)	**Effect (%change)**
Adrenal	0.05±0.005	0.05±0.004	p=0.75	0.05±0.002	0.08±0.028	p=0.29
Brain	4.21±0.4	4.17±0.1	p=0.91	4.42±0.3	6.03±1.8	p=0.44
Heart	6.48±0.4	6.40±0.2	p=0.85	6.37±0.2	9.43±3.4	p=0.42
Kidneys	5.33±0.2	4.95±0.3	p=0.29	5.44±0.2	7.75±2.0	p=0.32
Liver	24.29±0.6	22.15±0.7*	9%↓ p=0.046	22.65±1.1	34.78±8.7	p=0.24
Lungs	13.21±2.4	14.07±0.4	p=0.73	15.70±1.1	23.87±8.0	p=0.37
Pancreas	0.51±0.04	0.55±0.07	p=0.65	0.53±0.08	0.59±0.30	p=0.86
Spleen	4.11±0.3	4.36±0.2	p=0.55	4.13±0.7	6.05±2.1	p=0.44
Thymus	1.70±0.2	1.85±0.2	p=0.51	1.48±0.3	3.21±1.9	p=0.43
Thyroid	0.08±0.01	0.08±0.01	p=0.95	0.08±0.02	0.09±0.03	p=0.82

Table 3.6 Lamb organ weights, relative to body weight (g/kg) at 28 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Values are mean ± SEM. Control and canola groups were compared by an unpaired t-test for each gender. Significant differences are denoted by directional arrows.

3.4.5 Lamb adipose tissue weights

'Total' adipose tissue refers to the sum of the 4 depots sampled (omental, pericardial, perirenal and sternal). Mean relative sternal weights are not given for 7 days due to the small numbers of animals for which this data was collected. Total adipose tissue was therefore not calculated for this time point.

There were no significant differences in weights of any of the sampled adipose tissue, relative to body weight (grams per kilogram) between control and canola groups at 7 days. At 28 days, canola group males showed no differences to control males, however, females in the canola group showed a 12% decrease (p=0.036) in relative pericardial adipose tissue weight and a 25% decrease (p=0.025) in relative sternal adipose tissue weight, compared

with female controls. All adipose tissue depot weight data is shown in Table 3.7 and Table 3.8.

	Females			Males		
	Control (n=6)	Canola (n=6)	P value	Control (n=4)	Canola (n=4)	P value
Omental	2.64±0.3	3.21±0.5	p=0.39	3.53±0.6	3.02±0.2	p=0.42
Pericardial	1.27±0.08	1.33±0.13	p=0.67	2.30±0.63	1.22±0.05	p=0.21
Perirenal	7.86±1.1	8.96±1.7	p=0.61	9.05±1.4	6.06±1.5	p=0.19
Sternal	NA	NA	NA	NA	NA	NA
Total	NA	NA	NA	NA	NA	NA

Table 3.7 Lamb adipose tissue depot weights relative to body weight (g/kg) at 7 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Values are mean ± SEM. Control and canola groups were compared by an unpaired t-test for each gender.

	Females			Males		
	Control (n=7)	Canola (n=7)	**Effect (% change)**	Control (n=3)	Canola (n=3)	**Effect (% change)**
Omental	10.75±1.1	11.83±0.7	p=0.41	7.83±2.4	11.23±2.2	p=0.36
Pericardial	1.78±0.1	1.56±0.1*	12%↓ p=0.036	1.29±0.1	2.51±1.1	p=0.35
Perirenal	14.74±1.8	13.23±0.9	p=0.46	8.20±2.5	13.44±2.0	p=0.18
Sternal	6.8±0.6	5.1±0.3*	25%↓ p=0.025	3.4±1.1	7.4±1.8	p=0.21
Total	35.85±2.8	31.69±0.8	p=0.16	18.17±6.4	34.56±6.9	p=0.20

Table 3.8 Lamb adipose tissue depot weights relative to body weight (g/kg) at 28 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Values are mean ± SEM. Control and canola groups were compared by an unpaired t-test for each gender. Significant differences are denoted by directional arrows.

3.4.6 Gene expression in the perirenal adipose tissue of female lambs

Due to the absence of several tissue samples for male lambs, which would have left just two lambs in some groups, it was felt that it would not be possible to get meaningful mRNA expression results and interpretations from male lambs. Therefore, all gene expression, protein abundance and histology results are for female lambs only.

3.4.6.1 BAT/thermogenic gene expression

BAT/thermogenic gene expression in the perirenal adipose tissue of female lambs in the control and canola groups, at both 7 and 28 days are shown in Figure 3.10.

At 7 days, mean *UCP1* mRNA expression in the canola group was 50% lower than that of the control group, however, this was not statistically significant (p=0.067). At 28 days, there was also a decrease in *UCP1* mRNA expression in the canola group, in this case, 69%, however, again it was not significant (p=0.075). *ADRB3*, *CIDEA*, *DIO2* and *BMP7* mRNA expression did not differ significantly between groups.

Despite the lack of statistical significance, there seemed to be an overall trend that the expression of BAT/thermogenic genes was lower in the canola group compared to controls.

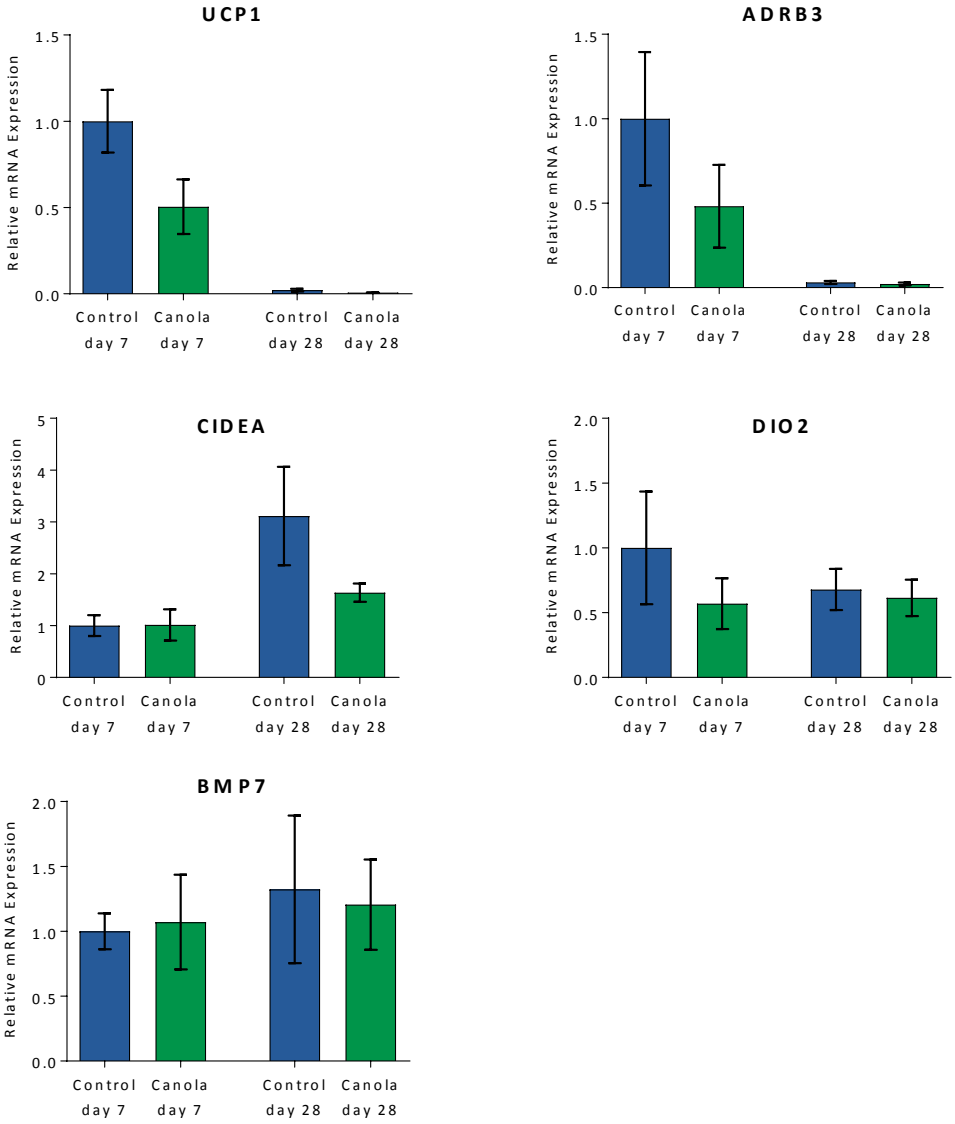

Figure 3.10 Relative mRNA expression of BAT/thermogenic genes in perirenal adipose tissue of female lambs by dietary group at 7 and 28 days.

Dietary group relates to maternal diets, which are control (standard diet, 7 days, n=6; 28 days, n=6) and canola (standard diet +3% canola oil, 7 days, n=6; 28 days, n=7). Values are mean ± SEM. Relative expression is normalised to the reference genes IPO8 and RPO. Control and canola groups were compared at each time point by unpaired t-test.

3.4.6.2 Expression of genes associated with white/brite adipose tissue

The mRNA expression of genes tested which are associated with WAT/brite adipose tissue are shown in Figure 3.11.

Leptin (a gene associated with WAT), showed no significant difference between dietary groups at 7 days, and although there appeared to be a decrease in the canola group at 28 days, this was not statistically significant (p=0.12). *RIP140*, known to suppress brite adipocyte development, showed wide variation within groups and there was no effect of dietary group. The two brite adipose tissue associated genes, *HOXC9* and *SHOX2*, also showed no effect of maternal diet during lactation.

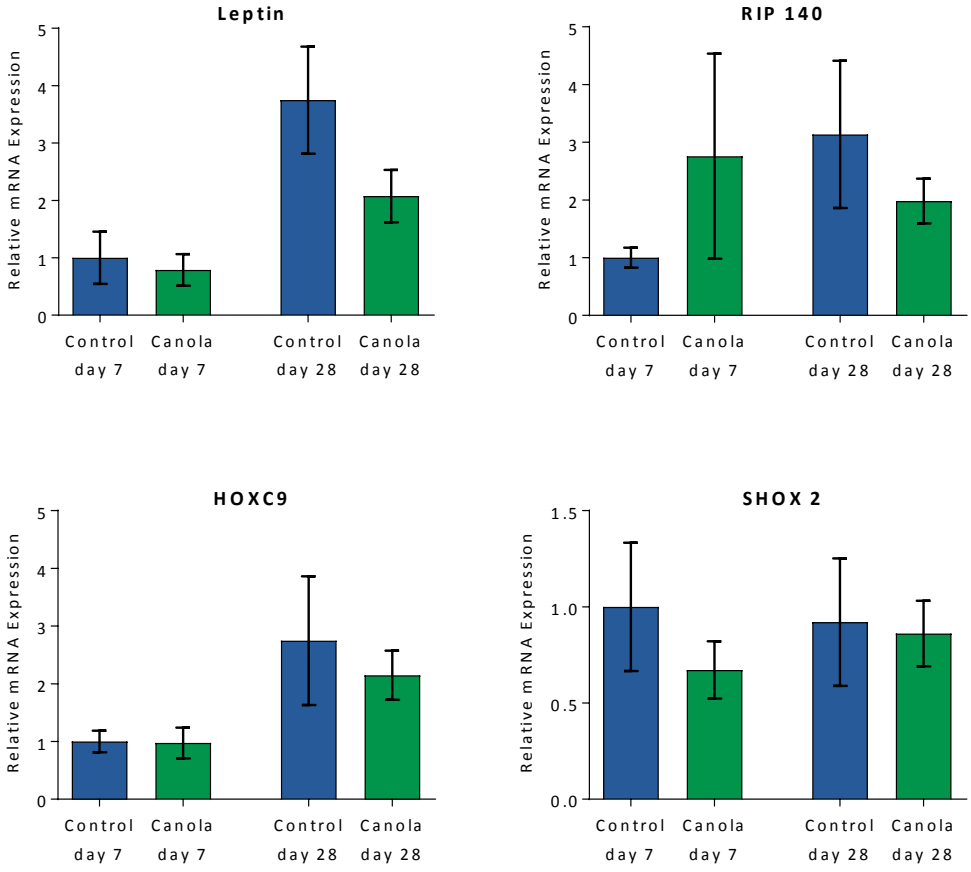

Figure 3.11 Relative mRNA expression of WAT/brite adipose tissue associated genes in perirenal adipose tissue of female lambs by dietary group at 7 and 28 days.

Dietary group relates to maternal diets, which are control (standard diet, 7 days, n=6; 28 days, n=6) and canola (standard diet +3% canola oil, 7 days, n=6; 28 days, n=7). Relative expression is normalised to the reference genes IPO8 and RPO. Values are mean ± SEM. Control and canola groups were compared at each time point by unpaired t-test.

3.4.7 UCP1 abundance in the perirenal adipose tissue of female lambs

Western blots were performed on the perirenal adipose tissue of female lambs in the control and canola groups, sampled at 7 or 28 days, using UCP1 antibodies. There were no effects of dietary group on the relative UCP1 at either day 7 (Figure 3.12) or 28 (Figure 3.13).

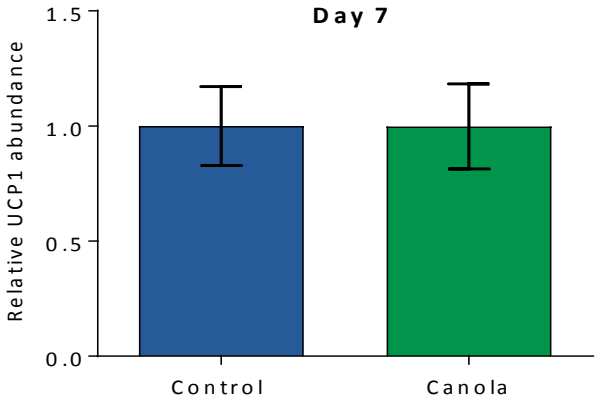

Figure 3.12 Relative UCP1 abundance in perirenal adipose tissue of female lambs at 7 days, by dietary group.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil), n=6 per group. Values are mean ± SEM.

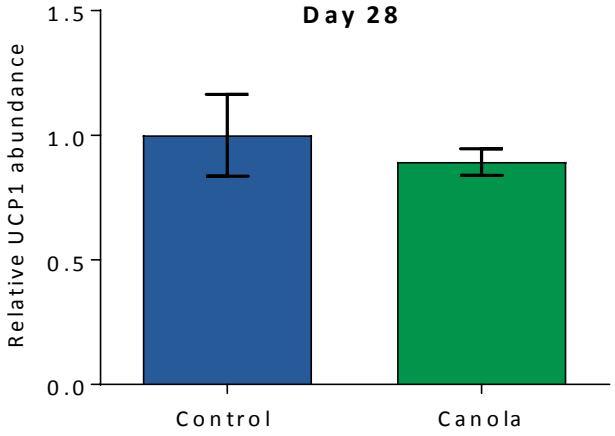

Figure 3.13 Relative UCP1 abundance in perirenal adipose tissue of female lambs at 28 days, by dietary group.

Dietary group relates to maternal diets, which are control (standard diet, n=4) and canola (standard diet +3% canola oil, n=6). Values are mean ± SEM.

3.4.8 Histological analysis of female lambs' perirenal adipose tissue

Sections of perirenal adipose tissue of 28 day old females were imaged in order to determine whether the maternal dietary intervention had an effect on the morphological appearance and UCP1 content of the perirenal adipose tissue depot.

3.4.8.1 Haematoxylin and eosin

Haematoxylin and eosin (H&E) staining enabled the tissues morphological structure to be examined, and compared between groups. The tissue morphology appeared consistent with that usually seen for this depot at this age in sheep[134] and there were no observable changes in the tissue appearance between the two dietary groups. A representative sample of tissue from each group is shown in Figure 3.14.

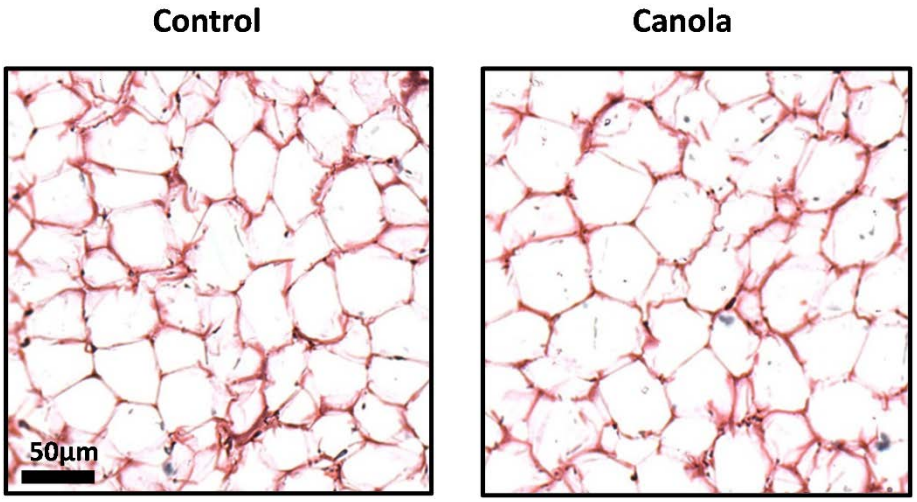

Figure 3.14 Representative haematoxylin and eosin (H&E) stained microscopic sections of perirenal adipose tissue, sampled at 28 days, at 20x magnification, on a Leica DMRB microscope.

Control: a female lamb whose mother was fed a standard diet. Canola: a female lamb whose mother was fed a standard diet + 3% canola oil.

3.4.8.2 Immunohistochemistry

Sections of perirenal adipose tissue of 28 day females were stained with a UCP1 antibody (methodology detailed in Chapter 2, Section 2.5.4) in order to determine whether UCP1 was present, and to compare the control and canola groups. The presence of UCP1 would be indicated by a brown stain. There was little evidence of the presence of UCP1 in the tissue (despite the positive controls staining for UCP1) and no apparent variation between the two groups. A representative sample of tissue from each group is shown in Figure 3.15.

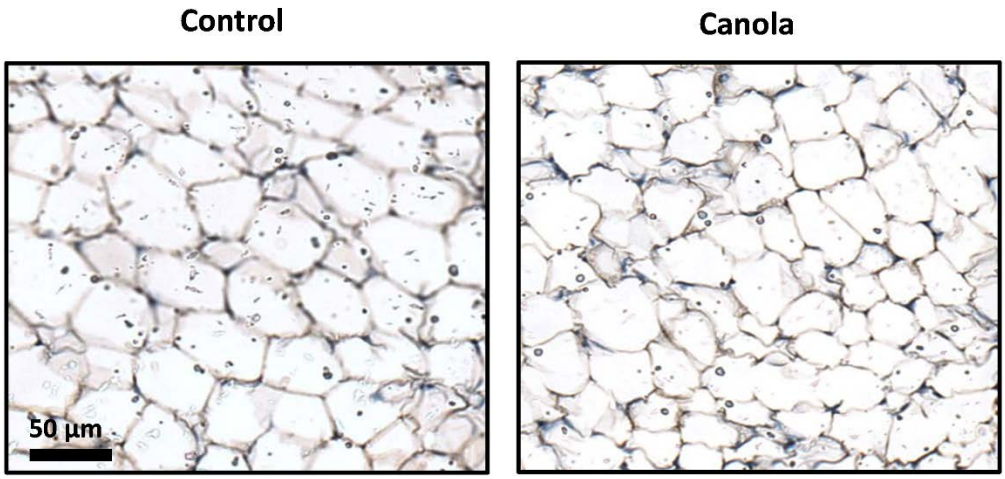

Figure 3.15 Representative uncoupling protein (UCP) 1 (1:750 primary antibody dilution) stained microscopic sections of perirenal adipose tissue, sampled at 28 days, at 20x magnification, on a Leica DMRB microscope.

Control: a female lamb whose mother was fed a standard diet. Canola: a female lamb whose mother was fed a standard diet + 3% canola oil. Presence of UCP1 is indicated by a brown stain (not present).

3.5 Discussion

3.5.1 Maternal supplementation had few effects on milk fatty acid profile

This study showed few effects of canola oil supplementation on milk fatty acid profile, with the exceptions being some changes in the proportions of certain omega-6 fatty acids, SFAs and certain, but not the hypothesised, CLA isomers, which are discussed further on. The reasons for the lack of effect of supplementation on milk fatty acids are somewhat unclear and go against the findings of other studies in ruminants[406,407,410,455] and indeed my subsequent study (Study B, Chapter 4). Potential causes for the variation in results will be discussed in (Chapter 4), but in brief, the lack of effect of supplementation in Study A may be attributable to the breed, parity or pre study diet of the sheep, all of which may have varied between the sheep in Study A and B.

3.5.1.1 Decreased milk linoleic acid may be responsible for decreased offspring adiposity

The decrease in omega-6 PUFAs is likely to be largely attributable to the decrease in LA (C18:2 n-6), as LA is the most abundant omega-6 PUFA. A study by DePeters *et al.*[456] also found a decrease in LA with canola oil supplementation in cows. The decrease in LA is likely to be due to its biohydrogenation in the rumen, as when De Peters *et al.*[456] infused LA abomasally (bypassing the rumen), the decrease was ameliorated and an increase in milk LA was observed[457]. My study also found an increase in stearic acid (C18:0), the end product of LA biohydrogenation[458], which further supports this proposed mechanism.

Omega-6 fatty acids are thought to contribute to adipose tissue development in early life[286]. The decrease in omega-6 PUFAs induced by canola oil supplementation may serve as at least a partial explanation for why female lambs in the canola group showed a decrease of adipose tissue weight in the sternal and pericardial depots. However, this does not explain why this decrease was not seen in males. It is known that in humans, females possess more adipose tissue than males regardless of their BMI, ethnicity or age[459]. This is also the case in sheep[460]. It is thought that these gender differences could, at least in part, be attributed to sex hormones, with post-menopausal women experiencing significant changes in adipose tissue distribution[461]. It has also been suggested that adult males have a less efficient conversion of α-Linolenic acid (ALA) to eicosapentaenoic acid (EPA) than females[462], and, whilst these are omega-3 fatty acids, omega-6 fatty acids use the same

elongase and desaturase enzymes, so potentially males and females may have different LA conversion rates, which may affect adipose tissue growth.

3.5.1.2 Canola oil supplementation did not change to total CLA content, with some increases in endogenously synthesised CLA isomers

The CLA present in the milk of ruminants can originate from two potential sources: from the rumen, where it is derived from LA; and from endogenous synthesis of vaccenic acid by mammary *delta*-9 desaturase[189,190] in the mammary gland. In this study I observed a decrease in LA, but this is unlikely to be solely responsible for the lack of CLA increase. The increase in stearic acid suggests that LA was biohydrogenated (to stearic acid) without being converted to CLA. In ruminant animals, the rumen is a formidable barrier against the delivery of unsaturated fatty acids to the intestines for absorption. This is due to a complex process in the rumen involving unsaturated fatty acids being hydrolysed to free fatty acids, which then undergo biohydrogenation, resulting in more SFAs arriving in the small intestine[463]. Duodenal flow-through of LA has been found to be particularly low (7%) in cows fed rapeseed oil (which is similar to canola oil)[464] especially when compared with rates of 64% in cows fed whole soybeans and tallow[465]. Therefore, it is not particularly surprising that I did not see an increase in LA in canola supplemented ewes' milk, however, a biological explanation for the observed decrease is a little more complex. A study on goats by Mir *et al.*[410] found a mean LA content of 4.4 % of fat in control animals, then 3.9% of fat with the addition of 2% canola oil, then 3.9% fat with 4% canola oil and finally 4.4% of fat with 6% canola oil. It is important to note that these results were not significantly different, however, it is interesting that this offers some support for a theory that was of Beam *et al.* that the concentration of lipid supplements is a major factor affecting biohydrogenation[466] as higher, but not lower, concentrations of fatty acids in the rumen altered biohydrogenation[466].

Biohydrogenation of fatty acids such as LA takes place in a two-part process. First, the dienoic acid is converted to a monoenoic acid, which is then converted to a SFA, such as stearic acid. Harfoot *et al*. suggested that higher concentrations of LA may interfere or 'compete' with the second part of the process, leading to accumulation of monoenoic acids and a decrease in the corresponding SFA[467]. CLA is formed as an intermediate product between the two parts of the process[468]. Therefore, it is possible that canola oil supplementation at lower levels, initially, increases (or does not affect) ruminal biohydrogenation of LA, resulting in a reduction of LA in the milk, with higher levels

inhibiting the secondary step of LA biohydrogenation, increasing CLA. However, a more detailed biological explanation of this effect is not possible within the parameters of this investigation. This does not explain why I found different effects in Study B (Chapter 4), despite sheep being given the same canola oil supplement at the same dose. It is possible that differences between the sheep used in the two studies, such as breed[469] or parity[470], affected biohydrogenation.

A study by Lee *et al.*[471] in 2011 used labelled LA in mixed ruminal microorganism cultures to trace carbon transfer to CLA, in order to investigate which isomers could be formed by the biohydrogenation of LA. The study found that 8 CLA isomers could be positively identified in the cultures. These were *cis*-9, *trans*-11; *trans*-10, *cis*-12; *trans*-9, *cis*-11; *trans*-9, *trans*-11; *trans*-11, *trans*-13; *trans*-8, *trans*-10; *cis*-9, *cis*-11 and *cis*-10, *cis*-12. There were no increases in any of these isomers in this study. Interestingly, I found an increase in *trans*-7, *cis*-9 CLA, which is said to be derived, predominantly, from endogenous synthesis via *delta*-9 desaturase[472]. The study by Lee *et al*. [471], in fact, did not identify any of the isomers which were increased in my study, suggesting they are not formed by ruminal biohydrogenation. This leaves me postulating whether the effect of canola oil, was through *delta*-9 desaturase activity, but ewes' ruminal synthesis and biohydrogenation of fatty acids was impaired.

The lack of change in the *cis*-9, *trans*-11 and total CLA levels with canola oil supplementation contradicts the findings of other research groups. The study by Mir *et al.*[410] discussed above also measured CLA levels in the milk, showing an increase in milk CLA with supplementation of 2% and 4% canola oil. The goats used for this study were in late lactation (187-191 days postpartum). This could be another potential explanation for the lack of results in my study, as the ewes in this study were in early lactation. It is known that the milk composition in early lactation differs from that in late lactation[473]. A similar study by Stanton *et al*.[474] found that cows supplemented with rapeseed oil showed increased milk CLA levels. This study looked at the effect of stage of lactation in cows on milk CLA levels, and found no effect. The stage of lactation for these animals ranged from 12-93 days and 99-193 days.

Another potential reason for this study not showing similar effects on milk composition as other published studies is of a slightly different nature, namely the much discussed 'positive publication bias' . This refers to research studies that have been conducted, but do not yield statistically significant results and/or fit the original hypothesis[475,476]. Researchers may not

try to publish such work, or it may be rejected by peer-reviewers or journal editors. Published studies suggest that CLA levels are subject to a large individual variation, even amongst sheep consuming the same diet[477], so it may be that some breeds or sheep of a certain age, stage of lactation, or who have had a certain number of lactations, do not respond to fatty acid supplementation as effectively as others. It may be that similar results have been found by other research groups, but they have not been published due to not fitting the hypothesis, or being thought of as 'not interesting'.

As previously mentioned, increases were observed in three CLA isomers in this study, *trans*-7, *cis*-9, *trans*-11, *trans*-13 and *trans*-10, *trans*-12, of 73%, 29% and 54% respectively in milk sampled at 28 days. The biological function of these isomers is largely unknown. There is a paucity of literature describing them and, whilst they have been previously identified and measured, no functional attributions have been made. The *trans*-7, *cis*-9 CLA isomer was first identified in 1998 by Yurawecz *et al.*[478]. The research group found this isomer present in milk, cheese, beef, human milk and adipose tissue. They found its concentration to range from 3% to 16% of total CLA, with more specific values of 6% in sheep milk, 3-5% in cow's milk and 6-10% in human breast milk. However, while this is interesting, in the absence of any information about the isomer's biological function, it is not possible to draw any conclusions.

3.5.1.3 Canola oil supplementation had minor effects on milk saturated fatty acids

Only two of the tested SFAs were affected by canola oil supplementation, in milk sampled at 28 days. There was a small (9%), yet significant, decrease in palmitic acid, and a 17% increase in stearic acid. From the existing literature, it may have been expected that a larger change in SFAs would be seen with supplementation, with studies in other ruminants showing that canola oil supplementation significantly decreases milk SFAs[406,407,479,480].

The stearic acid (C18:0) content of milk has been shown to be raised either by biohydrogenation of oleic, linoleic, and linolenic acids, as stearic acid is the end product of C18:0 PUFA biohydrogenation[481]. It is likely that upon entering the rumen, the C18:0 fatty acids present in canola oil, particularly oleic acid (C18:1 n-9)[482], become hydrogenated into stearic acid.

With regard to palmitic acid (C16:0), it has been observed that supplementation of plant oils can result in a shift towards 18:0, at the expense of 16:0, caused by the inhibitory effect

of long-chain fatty acids on mammary *de novo* fatty acid synthesis, which is responsible for approximately half of C16:0 in milk[483].

3.5.2 Effect of maternal supplementation on lamb's body and organ weights

There were no significant differences in lamb body weights or growth. This was to be expected as the maternal supplementation did not alter the total amount of fat present in their milk, which would be the most likely way for lamb body weight to be influenced in the short term. Maternal supplementation did not appear to affect the relative weight of the lambs' organs, with the only exception being a 9% decrease in liver weights of canola group females sampled at 28 days. This small change was not apparent at 7 days, or in males at either time point. There is little in the literature which helps to explain this. Previous research has shown an increase in the liver weight of 4 month old lambs fed an *ad libitum* diet[484], however, the lack of differences in body weight in my study indicates no substantial change in calorie intake. It is hard to attribute this liver change to any specific factor, due to this study largely being unsuccessful in altering milk composition. With few other phenotypic differences observed, it could be that this small, yet statistically significant, result was simply down to chance.

3.5.3 Effect of maternal supplementation on gene expression

The addition of canola oil to the maternal diet during lactation did not alter the mRNA or protein levels of UCP1 in the female lambs, nor did it have a clear effect on expression of any of the other genes tested. The tissue morphology was not notably different between lambs in the control and canola group. As supplementation yielded relatively few changes in the milk composition, especially in the CLA isomers which I hypothesised might affect BAT, it is unsurprising that there were no effects on offspring candidate gene expression. The gene expression of male lambs was not analysed in this study, due to small numbers. Therefore, I cannot rule out that their gene expression may have been affected by maternal supplementation.

3.6 Conclusion

In conclusion, results from this study showed neither what I expected, nor what I show in my research study detailed in Chapters 4 and 5. There are several reasons why this may be the case, and this highlights the complex systems which regulate milk lipids and shows that milk composition should not be considered as a standard entity.

It seems that CLA isomers which are known to undergo occur as a result of ruminal biohydrogenation, such as the *cis*-9, *trans*-11 isomer were largely unaffected in this study. On the other hand, some of those known to be generated by endogenous synthesis, such as the *trans*-7, *cis*-9, the *trans*-10, *trans*-12 and the *trans*-11, *trans*-13 CLA isomers, have increased. This leads me to speculate that there may have been factors influencing the ruminal biohydrogenation of the sheep in this study, which could potentially be linked to their genotype, or number of lactations.

The conclusion of this chapter is that supplementation of canola oil had unexpectedly few effects on milk fatty acid profile. However, it appears that even minor changes may affect offspring body composition. My study also suggests that some effects of the maternal supplementation on offspring adipose tissue weights are gender specific. The minor alterations in milk fatty acid profile had no effect on UCP1 abundance in the perirenal adipose tissue of female lambs.

3.7 Further investigation

Once it was determined that the study model was feasible, this study (Study A) was followed by another, similar study (Study B) which is detailed in Chapters 4 and 5. Study B also included an additional group supplemented with sunflower oil.

4 Manipulation of milk fatty acid profile by dietary fatty acid supplementation

4.1 Introduction

This study (Study B) is in two parts, summarised in Figure 4.1. The first part, covered in this chapter, investigated the effects of canola oil or sunflower oil supplementation on milk fatty acid profile in a ruminant, specifically sheep. The second part, covered in Chapter 5, investigated the effects of an altered milk fatty acid profile on the body composition, plasma metabolites and thermogenic capacity of adipose tissue in lambs.

Chapter 1 has discussed how early life nutrition is known to affect later health[485] and that breastfeeding has been linked in numerous studies to reduced risk of obesity later in life[216,256,486]. The exact mechanisms by which this protection is offered are unclear, it is likely to be due to a multitude of factors, one of which may be the specific nutritional composition of milk in early life.

Both human and ruminant milk fatty acid profiles are known to be altered by diet[487]. As discussed in Chapter 3, previous studies have found that the addition of canola oil to the diets of goats and cows increases milk CLA content[406,407,410]. Ruminant milk CLA content is known to vary depending on season[363], due to the associated changes in diet, with fresh grass being available in the summer[184]. As the majority of human dietary CLA comes from ruminant products such as milk and dairy[343], this is likely to mean that human dietary intake will also vary seasonally. CLA has a number of purported health benefits, most notably anticarcinogenic effects[293], reduction in body fat percentage[321] and reduced severity of atherosclerosis[488]. Of particular interest to my thesis is research suggesting that CLA could be involved in increasing the activity of BAT[330,333,334]. BAT is a potential target for the treatment and/or prevention of obesity[79] and it is therefore possible that the presence of fatty acids, such as CLA, in the early life diet could contribute to the protective effect of breastfeeding on obesity[256].

There is some research to suggest that the CLA content of infant formula is lower than that of human breastmilk[368,378], including my work in Chapter 6 . This apparent gap between CLA concentrations in infant formula and breast milk may be important, given that the aim of infant formula companies is to make infant formula as similar to breast milk as possible. The EFSA states that CLA must not be added to infant formula milk, however, the CLA that

is naturally present in milk fat is permitted to remain[489]. This makes supplementing the diet of animals producing the milk used in infant formula the most feasible option for increasing its CLA concentrations[368,404,408]. Dietary supplementation of dairy cows to improve the nutritional value of milk for adult consumption has been shown to be effective in increasing milk CLA[404]. However, to my knowledge, it has not been studied with regards to infant formula.

4.2 Supplements used for investigation

Canola oil and sunflower oil were selected for this study due to their high unsaturated fatty acid content (around 93% and 88% of fatty acids respectively[482]). The exact compositions of the oils utilised for ewe dietary supplementation in my study are not known and reported compositions do vary[490]. Table 4.1 shows the reported fatty acid composition of canola and sunflower oil in a study by Zambiazi *et al*.[482]. Regardless of minor differences in composition, canola oil is higher in MUFAs and lower in PUFAs than sunflower oil[482,490]. Studies investigating the effect of dietary supplementation on milk fatty acid profile have found that both canola oil and sunflower oil supplementation leads to decreased milk short and medium-chain SFAs, increased long-chain fatty acids and MUFAs and increases in *cis*-9, *trans*-11 CLA (See Table 1.5, Page 48, for a summary of previous studies). Ewes in my study were supplemented with 3% of canola/sunflower oil, based on other studies showing an effect on milk fatty acid profile in similar doses. In particular, a study in goats showed effects of 2% and 4% canola oil supplementation[410]. Higher concentrations appear to lead to reduced milk fat content[410].

%	Canola	Sunflower
Total Saturated	**6.98**	**12.36**
C14:0	0.06	0.06
C16:0	3.75	5.7
C17:0	0.04	0.04
C18:0	1.87	4.79
C20:0	0.64	0.30
C22:0	0.35	1.16
C24:0	0.27	0.31
Unsaturated		
Total MUFA	**64.42**	**15.93**
Total PUFA	**28.60**	**71.71**
C16:1	0.21	-
C17:1	-	0.06
C18:1	62.41	15.26
C18:2	20.12	71.17
C18:3	8.37	0.45
C20:1	1.54	0.22
C20:2	0.11	0.09
C24:1	0.26	0.39

Table 4.1 Composition of canola and sunflower oil based on data from Zambiazi *et al.*[482].

The first number following the 'C' for each fatty acid refers to the number of carbons it has. The number following the colon refers to the number of double bonds. MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

4.3 Hypotheses

I hypothesised that supplementation of the diet of lactating ewes with 3% canola or sunflower oil would result in the following effects on milk fatty acid profile:

- a decrease in SFAs.
- an increase in MUFAs.
- an increase in total and *cis*-9, *trans*-11 CLA.

4.4 Methods

A detailed description of all the methods used in this chapter can be found in Chapter 2. The experimental model used in this chapter is summarised in Figure 4.1. All reported animal experimentations for this study were performed by Professor Michael Symonds, Dr Mark Birtwistle, Dr Viv Perry and Mr Graeme Davies at the University of Nottingham, Sutton Bonington campus. The sheep milk samples were sent to The University of Pecs, Hungary to be analysed by Dr Tamás Marosvölgyi. A full description of techniques used for gene expression, histological and protein analyses are detailed in Chapter 2, along with a description of statistical analysis performed (Section 2.7, Page 94).

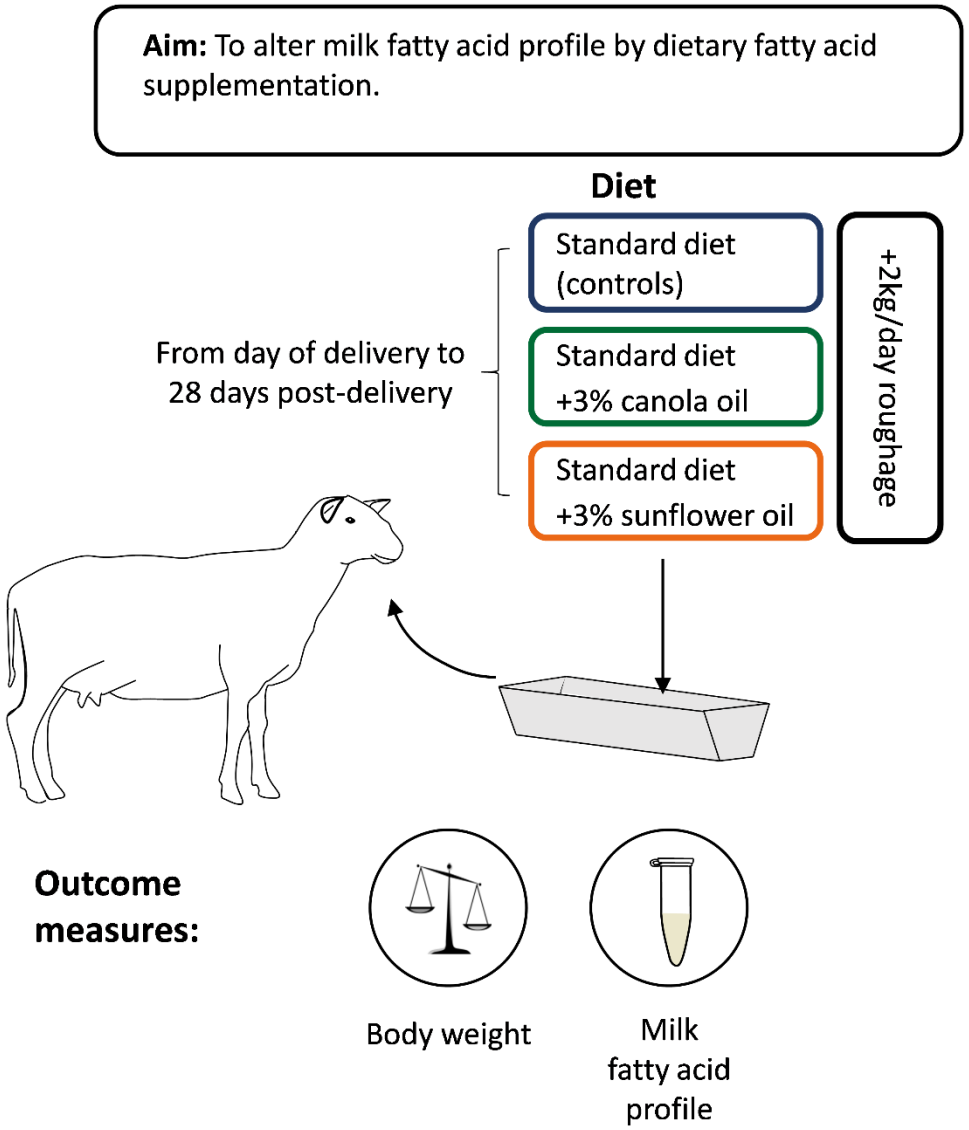

Figure 4.1 Summary of sheep investigation in Chapters 4.

Unfortunately, milk analysis was not possible for all of the ewes in this study. At day 7, milk could not be obtained from 2 ewes in the control group, 3 in the canola group and 3 in the sunflower group. At day 28, milk could not be obtained from 1 ewe in the control group, 1 ewe in the canola group and 2 ewes in the sunflower group. All collected samples were analysed for CLA and its isomers. Other milk fat analyses (total fat, SFAs, MUFAs, omega-3, -6, and -9 fatty acids and TFAs), were performed in a separate laboratory, some further samples were unable to be analysed due to either contamination or insufficient sample quantity. This meant that analysis was not completed for a further 3 ewes in the control group, 1 in the canola group and 3 in the sunflower group, at day 7, and 2 in the canola group and 2 in the sunflower group at day 28. This meant that final numbers for the fat analysis (excluding CLA) were very low in the sunflower group at 7 days (n=3). As this must be considered when interpreting the results of this study, animal sample numbers are presented alongside each result.

4.5 Results

4.5.1 The effects of maternal fatty acid supplementation on milk fatty acid profile in the first month of lactation in sheep

4.5.1.1 Total fat

Maternal dietary supplementation of canola or sunflower oil had no overall effect on the total fat content of the milk, at either 7 or 28 days, as shown in Figure 4.2.

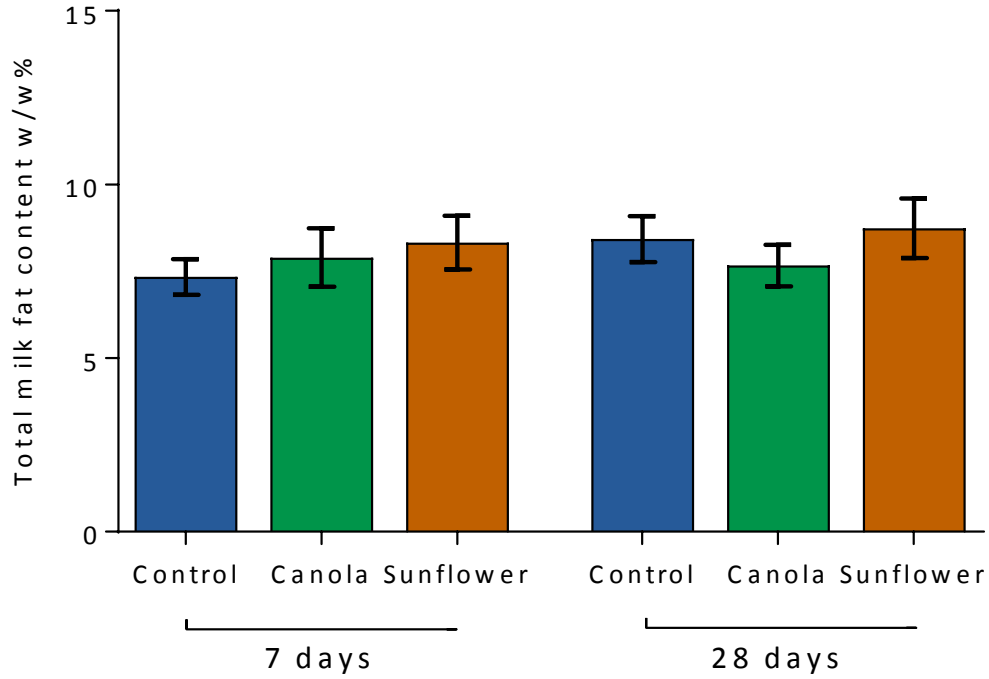

Figure 4.2 Milk fat content (w/w% - percentage by weight of total solution) at 7 and 28 days of lactation.

Samples from ewes fed a standard diet (control; day 7 n=6, day 28 n=8), a standard diet + 3% canola oil (canola; day 7 n=5, day 28 n=6), or a control diet + 3% sunflower oil (sunflower; day 7 n=3, day 28 n=5). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. There were no significant differences (p≥0.05).

4.5.1.2 Saturated Fatty Acids

Milk was analysed for concentrations of SFAs. Results are summarised in heat maps (Figure 4.3) and tabulated in Table 4.2.

The medium-chain (7-12 carbons long) fatty acids caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0) all decreased by approximately 40-50% by day 7 in the canola

group's milk, compared to controls, but were not significantly lower in the sunflower group at this time point. By 28 days, these medium-chain fatty acids were still lower in the milk of canola supplemented ewes and had decreased, but to a lesser extent, in the milk of sunflower supplemented ewes. Long-chain (≥12 carbons long) fatty acids myristic acid (C14:0) and palmitic acid (C16:0) both showed lower concentrations in the milk of canola and sunflower supplemented ewes at each time point (Table 4.2). The medium-chain undecylic acid (C11:0) and long-chain tridecylic acid (C13:0) milk concentrations were only affected by canola oil supplementation, showing decreases at both 7 and 28 days (Table 4.2). In contrast to the other SFAs, increases in long-chain fatty acids, stearic (C18:0) and arachidic acid (C20:0) were seen in the milk of canola supplemented ewes at 7 days and in both canola and sunflower ewes' milk at 28 days. Finally, milk concentrations of the long-chain behenic acid (C22:0) were elevated at both 7 and 28 days in the milk of the sunflower oil supplemented group only.

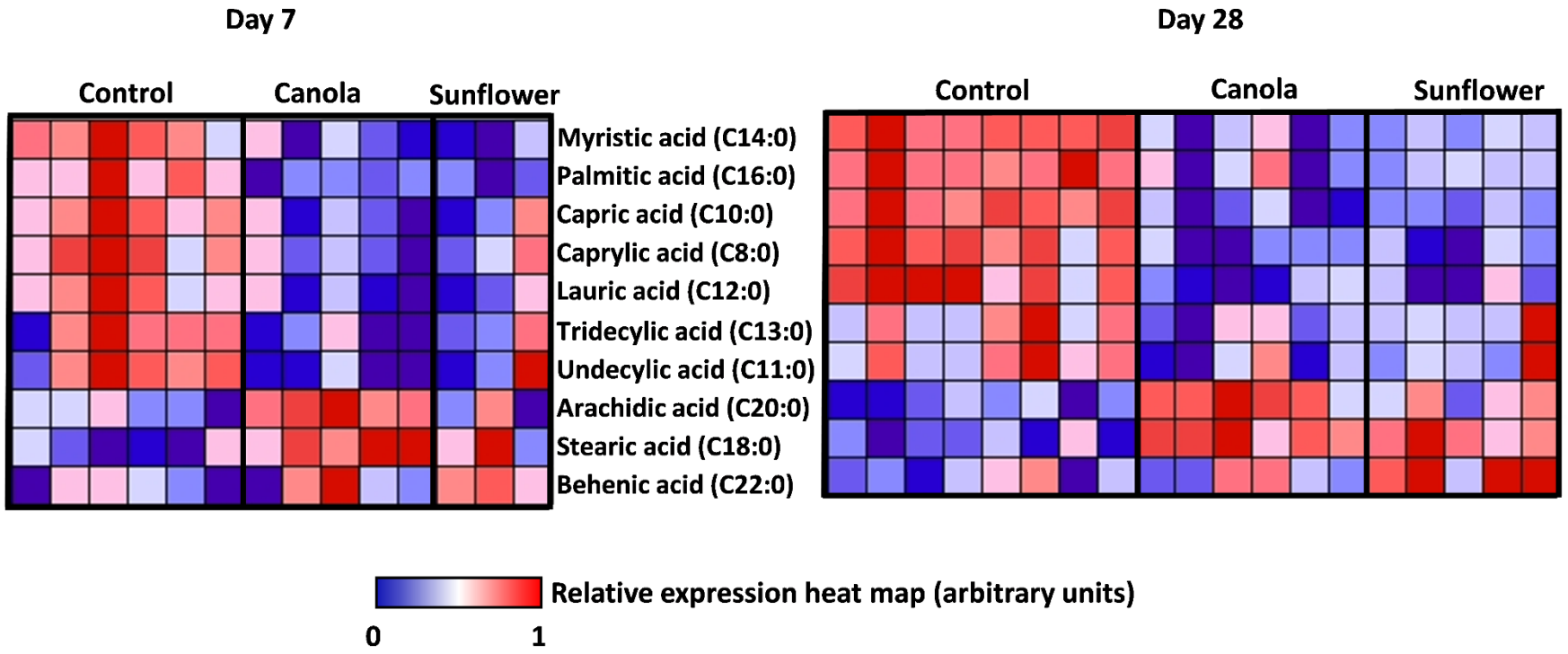

Figure 4.3 Heat map showing relative saturated fatty acid levels present in milk sampled at 7 and 28 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Fatty acid relative quantities are visualised by a colour scale, which ranges from bright red (highest value) through to dark blue (lowest value), see legend. Each square represents an individual sample. Heat map makes comparisons between the dietary groups at each separate time point independently and is not designed to compare the two time points. Heat maps were generated using GenePattern software (Broad Institute, MA, USA).

Fatty acid	Common name	7 days				28 days			
		Control (n=6)	Canola (n=5)	Sunflower (n=3)	Effect of supplement (% change)	Control (n=8)	Canola (n=6)	Sunflower (n=5)	Effect of supplement (% change)
C8:0	**Caprylic Acid**	2.52±0.19	1.47±0.24**	1.90±0.35	42%↓ p=0.0065 ↔p=0.13	2.92±0.07	2.12±0.23**	2.23±0.06****	27%↓ p=0.0028 24%↓ p<0.0001
C10:0	**Capric Acid**	5.85±0.63	2.79±0.51**	3.55±0.90	52%↓ p=0.005 ↔p=0.	8.37±0.22	4.91±0.59****	5.60±0.17****	41%↓ p<0.0001 33%↓ p<0.0001
C11:0	**Undecylic Acid**	0.057±0.010	0.016±0.005**	0.038±0.022	72%↓p=0.0056 ↔p=0.	0.080±0.010	0.042±0.011*	0.067±0.018	48%↓ p=0.028 ↔p=0.
C12:0	**Lauric Acid**	3.19±0.35	1.85±0.23*	1.98±0.34	42%↓ p=0.013 ↔p=0.	4.80±0.2	2.67±0.2***	3.00±0.1****	44% ↓ p<0.0001 38%↓ p<0.0001
C13:0	**Tridecylic Acid**	0.060±0.01	0.032±0.01*	0.043±0.01	47%↓ p=0.016 ↔p=0.	0.087±0.01	0.057±0.01*	0.079±0.02	34%↓ p=0.0499 ↔p=0.
C14:0	**Myristic Acid**	7.42±0.5	5.26±0.4**	4.94±0.4*	29%↓ p=0.008 33%↓ p=0.013	10.87±0.4	7.60±0.4****	7.73±0.4***	30%↓ p<0.0001 29%↓ p=0.0002
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Fatty acid	Common name	7 days				28 days			
		Control (n=6)	Canola (n=5)	Sunflower (n=3)	Effect of supplement (% change)	Control (n=8)	Canola (n=6)	Sunflower (n=5)	Effect of supplement (% change)
C16:0	**Palmitic Acid**	22.49±0.8	19.27±0.2*	18.83±0.4*	14%↓ p=0.014 16%↓ p=0.035	27.93±0.9	21.71±0.7***	21.71±1.0***	22%↓p=0.0002 22%↓p=0.0008
C18:0	**Stearic Acid**	13.06±0.7	17.55±0.7**	16.02±1.5	34%↑p=0.0017 ↔p=0.38	9.38±0.6	14.60±0.6***	14.20±0.7***	56% ↑ p=0.0001 51%↑ p=0.0004
C20:0	**Arachidic Acid**	0.16±0.01	0.21±0.01**	0.15±0.02	31%↑p=0.0013 ↔p=0.73	0.19±0.01	0.28±0.01****	0.23±0.01*	47%↑ p<0.0001 21%↑ p=0.016
C22:0	**Behenic Acid**	0.059±0.002	0.064±0.005	0.068±0.002*	↔p=0.35 15 %↑ p=0.028	0.087±0.005	0.095±0.008	0.131±0.010**	↔p=0.39 51%↑ p=0.0015

Table 4.2 Milk saturated fatty acid content (% of fat) at 7 and 28 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Within sampling day, the mean of each treatment group was compared with corresponding controls, by an unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

4.5.1.3 Monounsaturated fatty acids

At 7 days of lactation, milk from ewes receiving a diet supplemented with canola oil showed a significant 24% increase in milk MUFAs (p=0.007, Figure 4.4). A similar increase was found in the sunflower group at this time point, but in this case the difference was not significant (p=0.054), likely to be due to the low sample size (n=3). By 28 days, both canola and sunflower group ewes showed significant increases in milk MUFA levels of 50 and 44% respectively (both p<0.0001).

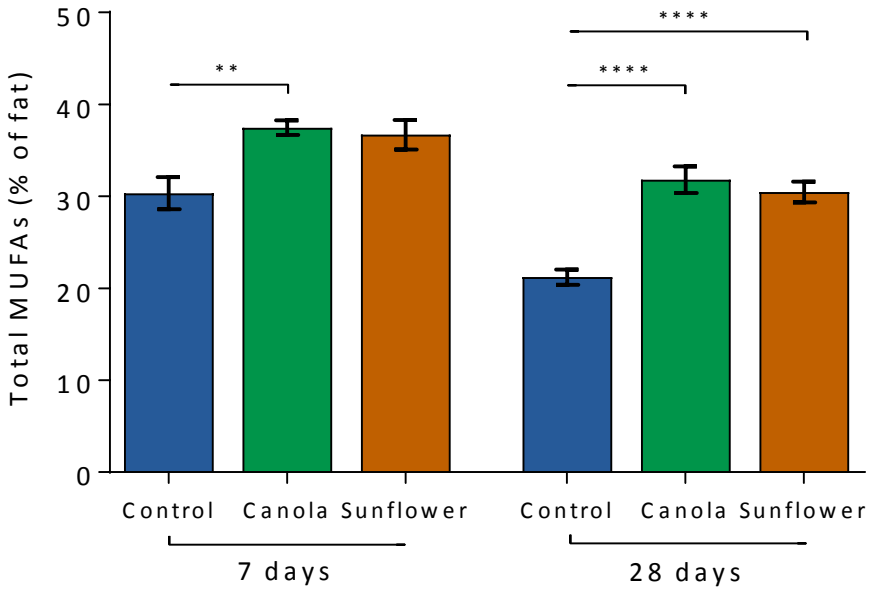

Figure 4.4 Milk monounsaturated fatty acid (MUFA) content (% of fat) at 7 and 28 days of lactation.

Ewes fed a control diet (control; day 7 n=6, day 28 n=8), a control diet + 3% canola oil (canola; day 7 n=5, day 28 n=6) or a control diet + 3% sunflower oil (sunflower; day 7 n=3, day 28 n=5). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by **p=0.007, ****p<0.0001.

For the individual MUFA analysis, at 7 and 28 days, all data is shown in Table 4.3.

Oleic acid (C18:1 n-9) is by far the most abundant MUFA, and so followed a very similar trend to total MUFAs. It was significantly increased at 7 (24%, $p=0.007$) and 28 (53%, $p<0.0001$) days in the milk of canola oil supplemented ewes. The increase in the milk oleic acid content in the sunflower supplemented ewes was not statistically significant at 7 days, again, possibly due to the low sample size, but had reached statistical significance by 28 days (45%, $p<0.0001$). Data is shown in Figure 4.4.

Milk from ewes in the canola group also showed a significant increase (72%, $p<0.0001$) in erucic acid (C22:1 n-9) concentrations at 28, but not 7 days. Milk from sunflower supplemented ewes showed no significant differences from controls. Nervonic acid (C24:1 n-9) showed no significant changes at either time point, in either treatment group.

		Day 7				Day 28			
Fatty acid	Common name	Control (n=6)	Canola (n=5)	Sunflower (n=3)	Effect of supplement (% change)	Control (n=8)	Canola (n=6)	Sunflower (n=5)	Effect of supplement (% change)
C18:1 n-9	oleic acid	29.52±1.7	36.72±0.8**	35.98±2.7	24% ↑ p=0.007 ↔p=0.38	20.34±2.4	**** 31.03±3.5	**** 29.58±2.6	53% ↑ p<0.0001 45%↑ p<0.0001
C22:1 n-9	erucic acid	0.018±0.004	0.019±0.002	0.012±0.0001	↔p=0.90 ↔p=0.45	0.018±0.0008	**** 0.031±0.002	0.021±0.002	72%↑ p<0.0001 ↔p=0.12
C24:1 n-9	nervonic acid	0.012±0.001	0.014±0.001	0.0093±0.002	↔p=0.87 ↔p=0.36	0.018±0.005	0.020±0.007	0.017±0.002	↔p=0.55 ↔p=0.49

Table 4.3 Milk omega-9 monounsaturated fatty acid content (% of fat) at 7 and 28 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Within sampling day, the mean of each treatment group was compared with corresponding controls, by an unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

4.5.1.4 Omega-3 and -6 fatty acids

Omega-3 and -6 concentrations in the milk samples are summarised in heat maps (Figure 4.5) and data is shown in Table 4.4 and Table 4.5. Supplementation with canola or sunflower oil did not have any significant effects on milk omega-3 fatty acid concentrations, at either 7 or 28 days. Milk omega-6 levels, however, did appear to be more influenced by maternal diet. Omega-6 long-chain polyunsaturated fatty acids (LCPUFAs) showed a significant decline in both canola (27%, p=0.019) and sunflower (23%, p=0.038) at day 7, but by day 28 only the milk from ewes supplemented with canola oil had significantly less omega-6 LCPUFAs (28%, p=0.024).

In contrast, supplementation with canola oil had no effect on total omega-6 PUFAs at either time point, while milk from ewes supplemented with sunflower oil showed a significant increase in total omega-6 PUFAs at 28 days (26%, p=0.0056), but no change at 7 days. This is likely to be mainly attributable to the changes in the most abundant omega-6 PUFA, LA (C18:2 n-6), which showed a significant increase in the sunflower group at 28 days (31%, p=0.028), but not day 7, and was not different between control and canola at either time point.

At 7 days, milk arachidonic acid (C20:4 n-6) concentrations were significantly lower in the canola group (32%, p=0.019) and the sunflower group (26%, p=0.04) compared with controls. However, by 28 days, only the canola group showed a significant reduction in arachidonic acid (32%, p=0.0087). Milk docosadienoic acid (C22:2 n-6) concentrations were significantly lower in the canola group at 7 days (71%, p=0.03) and by 28 days, concentrations were lower than controls in both the canola group (68%, p=0.0015) and sunflower group (51%, p=0.017). Docosapentaenoic acid (C22:5 n-6) concentrations in the milk of the canola and sunflower group ewes were 39% (p=0.001) and 39% (p=0.009) lower respectively when compared to controls at 7 days. At 28 days, only the canola group retained a significant reduction (57%) in docosapentaenoic acid (p=0.007).

A group of omega-6 fatty acids all displayed a similar pattern, showing no significant changes at 7 days, but reduced concentrations in the milk of only the canola supplemented group, at 28 days. These omega-6 fatty acids were: γ-linolenic acid (C18:3 n-6) which showed a 37% reduction (p=0.002); eicosadienoic acid (C20:2 n-6) which showed a 27% (p=0.01) reduction; and adrenic acid (C22:4 n-6), which was 60% lower than controls (p=0.007). Finally, levels of dihomo- γ-linolenic acid (C20:3 n-6) were unaffected by canola or sunflower oil supplementation at either time point.

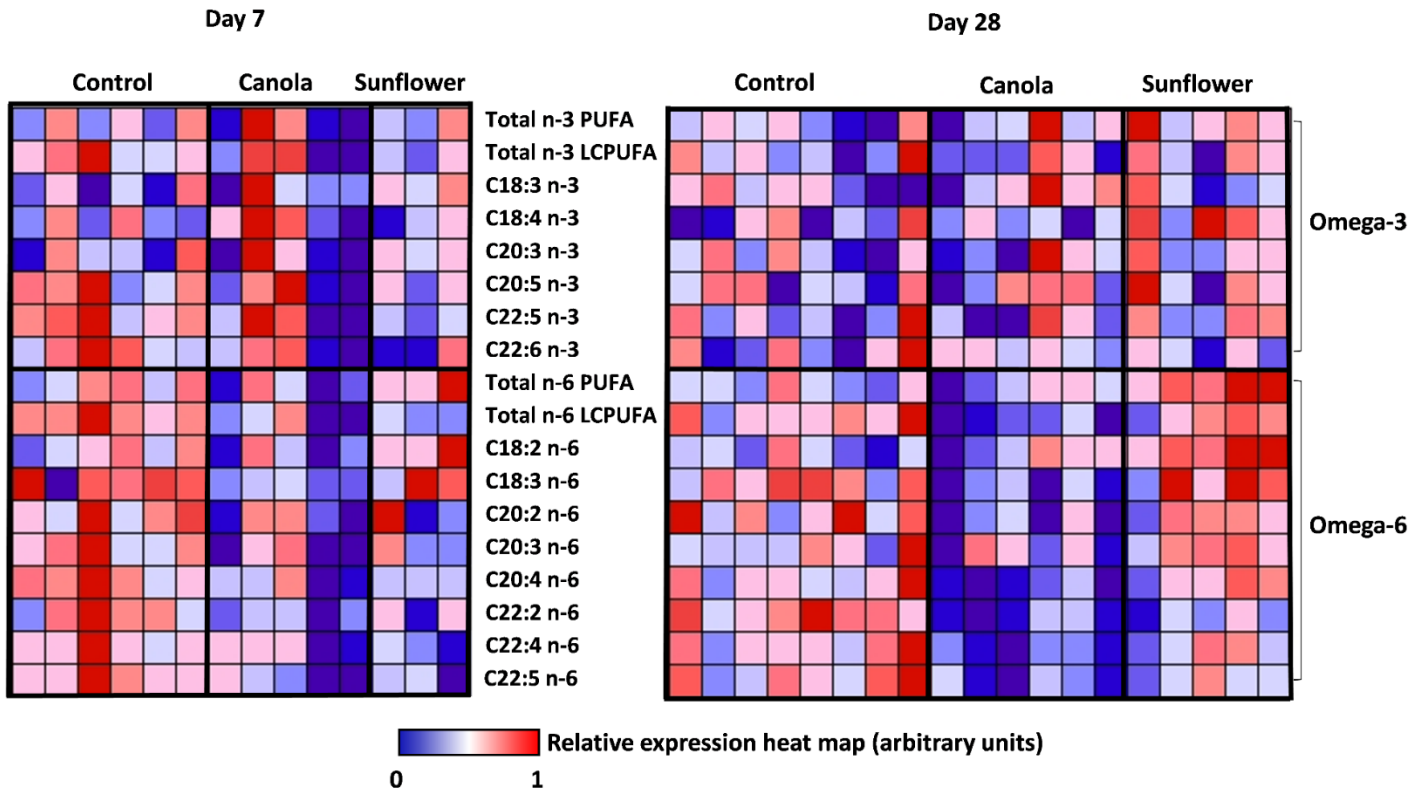

Figure 4.5 Heat map showing relative milk omega-3 and -6 fatty acid content at 7 and 28 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Fatty acid relative quantities are visualised by a colour scale, which ranges from bright red (highest value) through to dark blue (lowest value), see legend. Each square represents an individual sample. Heat maps were generated using GenePattern software (Broad Institute, MA, USA).

	Fatty acid	Common name	Control (n=6)	Canola (n=5)	Sunflower (n=3)	Effect of supplement (% change)
Omega-3	n-3 PUFA	Omega-3 PUFAs	1.19±0.07	1.231±0.21	1.172±0.09	↔ p=0.83, ↔ p=0.91
	n-3 LCPUFA	Omega-3 LCPUFAs	0.41±0.02	0.3494±0.05	0.3417±0.03	↔ p=0.26, ↔ p=0.11
	C18:3 n-3	α-Linolenic acid (ALA)	0.53±0.07	0.6±0.12	0.62±0.05	↔ p=0.65, ↔ p=0.44
	C18:4 n-3	Stearidonic acid	0.24±0.03	0.29±0.05	0.21±0.03	↔ p=0.45, ↔ p=0.54
	C20:3 n-3	Eicosatrienoic acid	0.01±0.001	0.01±0.002	0.01±0.0001	↔ p=0.97, ↔ p=0.98
	C20:5 n-3	Eicosapentaenoic acid (EPA)	0.1±0.006	0.08±0.013	0.09±0.008	↔ p=0.19, ↔ p=0.27
	C22:5 n-3	Docosapentaenoic acid	0.21±0.01	0.19±0.03	0.18±0.01	↔ p=0.63, ↔ p=0.69
	C22:6 n-3	Docosahexaenoic acid (DHA)	0.091±0.008	0.076±0.012	0.069±0.013	↔ p=0.32, ↔ p=0.18
Omega-6	n-6 PUFA	Omega-6 PUFAs	2.55±0.10	2.26±0.17	2.76±0.25	↔ p=0.15, ↔ p=0.38
	n-6 LCPUFA	Omega-6 LCPUFAs	0.3±0.02	0.22±0.02*	0.23±0.01*	27%↓ p=0.019. 23%↓ p=0.038
	C18:2 n-6	Linoleic acid (LA)	2.24±0.1	2.04±0.2	2.51±0.2	↔ p=0.27, ↔ p=0.26
	C18:3 n-6	γ-linolenic acid	0.04±0.005	0.02±0.002	0.04±0.005	↔ p=0.075, ↔ p=0.99
	C20:2 n-6	Eicosadienoic acid	0.05±0.003	0.04±0.004	0.05±0.008	↔ p=0.58, ↔ p=0.37
	C20:3 n-6	Dihomo- γ-linolenic acid	0.018±0.002	0.013±0.002	0.015±0.001	↔ p=0.28, ↔ p=0.37
	C20:4 n-6	arachidonic acid (AA)	0.19±0.01	0.13±0.02*	0.14±0.001*	32%↓ p=0.019, 26%↓ p=0.04
	C22:2 n-6	Docosadienoic acid	0.0007±0.0001	0.0002±0.0001*	0.0004±0.0001	71%↓ p=0.03, ↔ p=0.68
	C22:4 n-6	Adrenic acid	0.008±0.001	0.005±0.001	0.005±0.001	↔ p=0.09 , ↔ p=0.09
	C22:5 n-6	Docosapentaenoic acid	0.0044±0.0004	0.0027±0.0002**	0.0027±0.0003**	39%↓ p=0.001, 39%↓ p=0.009

Table 4.4 Milk omega-3 and 6 fatty acid content (% of fat) at 7 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Within sampling day, the mean of each treatment group was compared with corresponding controls, by an unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower). PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid.

	Fatty acid	Common name	Control (n=8)	Canola (n=6)	Sunflower (n=5)	Effect of supplement (% change)
Omega-3	n-3 PUFA	Omega-3 PUFAs	0.84±0.04	0.9064±0.08	0.9781±0.08	↔ p=0.49 , ↔p=0.12
	n-3 LCPUFA	Omega-3 LCPUFAs	0.34±0.02	0.3306±0.02	0.3496±0.02	↔ p=0.69 , ↔p=0.87
	C18:3 n-3	α-Linolenic acid (ALA)	0.35±0.03	0.4273±0.06	0.3814±0.05	↔ p=0.23, ↔p=0.56
	C18:4 n-3	Stearidonic acid	0.15±0.03	0.1485±0.02	0.2472±0.04	↔ p=0.92 , ↔p=0.063
	C20:3 n-3	Eicosatrienoic acid	0.0058±0.0005	0.006±0.001	0.0063±0.0007	↔ p=0.79 , ↔p=0.5
	C20:5 n-3	Eicosapentaenoic acid (EPA)	0.076±0.005	0.076±0.007	0.081±0.009	↔ p=0.97 , ↔p=0.60
	C22:5 n-3	Docosapentaenoic acid	0.2±0.01	0.19±0.01	0.21±0.01	↔ p=0.63, ↔p=0.69
	C22:6 n-3	Docosahexaenoic acid (DHA)	0.065±0.008	0.061±0.002	0.057±0.004	↔ p=0.71 , ↔p=0.49
Omega-6	n-6 PUFA	Omega-6 PUFAs	2.43±0.11	2.30±0.1	3.05±0.16**	↔ p=0.49, 26% ↑ p=0.0056
	n-6 LCPUFA	Omega-6 LCPUFAs	0.36±0.021	0.26±0.01**	0.35±0.02	28% ↓ p=0.024, ↔p=0.66
	C18:2 n-6	Linoleic acid (LA)	2.06±0.10	2.05±0.1	2.7±0.1**	↔ p=0.95 , 31% ↑ p=0.028
	C18:3 n-6	γ-linolenic acid	0.047±0.003	0.0297±0.003**	0.057±0.005	37% ↓ p=0.002, ↔p=0.54
	C20:2 n-6	Eicosadienoic acid	0.085±0.006	0.062±0.005*	0.077±0.005	27% ↓ p=0.01, ↔p=0.37
	C20:3 n-6	Dihomo- γ-linolenic acid	0.026±0.002	0.022±0.003	0.029±0.002	↔ p=0.28 , ↔p=0.37
	C20:4 n-6	arachidonic acid (AA)	0.19±0.01	0.13±0.006**	0.18±0.02	32% ↓ p=0.0087, ↔p=0.85
	C22:2 n-6	Docosadienoic acid	0.0015±0.0002	0.00048±0.0001**	0.00074±0.0001*	68% ↓ p=0.0015, 51% ↓ p=0.017
	C22:4 n-6	Adrenic acid	0.015±0.002	0.006±0.0009**	0.01±0.002	60% ↓ p=0.007, ↔p=0.36
	C22:5 n-6	Docosapentaenoic acid	0.007±0.0009	0.003±0.0004**	0.005±0.0005	57%↓ p=0.007, ↔p=0.13

Table 4.5 Milk omega-3 and 6 fatty acid content (% of fat) at 28 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Within sampling day, the mean of each treatment group was compared with corresponding controls, by an unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower). PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid.

4.5.1.5 Trans Fatty Acids

Maternal supplementation of canola and sunflower oil increased the TFA content of milk sampled at 7 days by 31% (p=0.007) and 47% (p=0.012) respectively, shown in Figure 4.6. By 28 days TFAs were increased by 80% in the canola group (p=0.0001) and 107% in the sunflower group (p=0.0002), compared to controls.

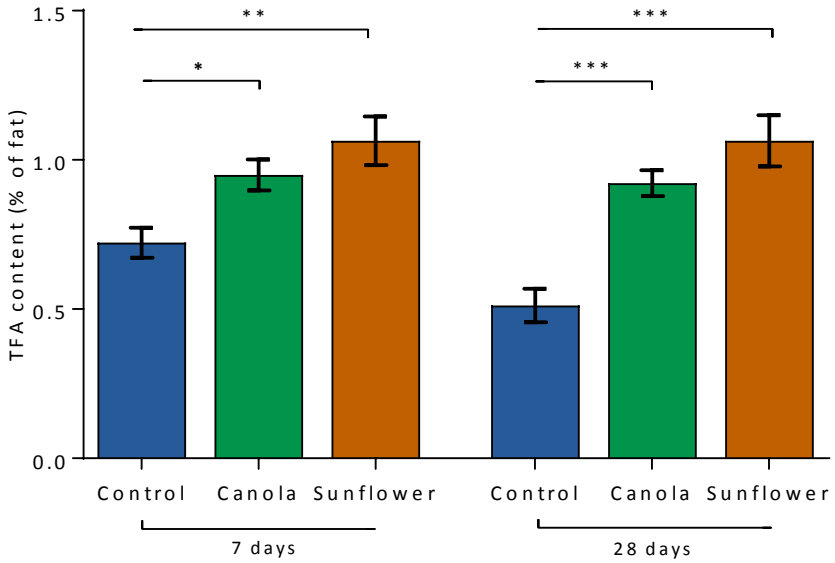

Figure 4.6 Milk t*rans* fatty acid content (% of fat) at 7 and 28 days of lactation.

Ewes fed a control diet (control; day 7 n=6, day 28 n=8), a control diet + 3% canola oil (canola; day 7 n=5, day 28 n=6) or a control diet + 3% sunflower oil (sunflower; day 7 n=3, day 28 n=5). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by *p=0.012, **p=0.007 ***p=0.0001 (control vs canola), p=0.0002 (control vs sunflower), compared by unpaired t-test.

4.5.1.6 Total CLA

Whilst there were no significant effects of dietary intervention on milk total CLA concentrations at 7 days, by 28 days the milk of canola group ewes had 75% more total CLA than controls ($p<0.0001$) . The milk of sunflower group ewes had 85% more total CLA than controls ($p<0.0001$, Figure 4.7). It is noteworthy that it appears that in controls, CLA decreases between 7 and 28 days. The addition of 3% sunflower or canola oil to the ewe's diet seems to prevent this decrease between the two time points.

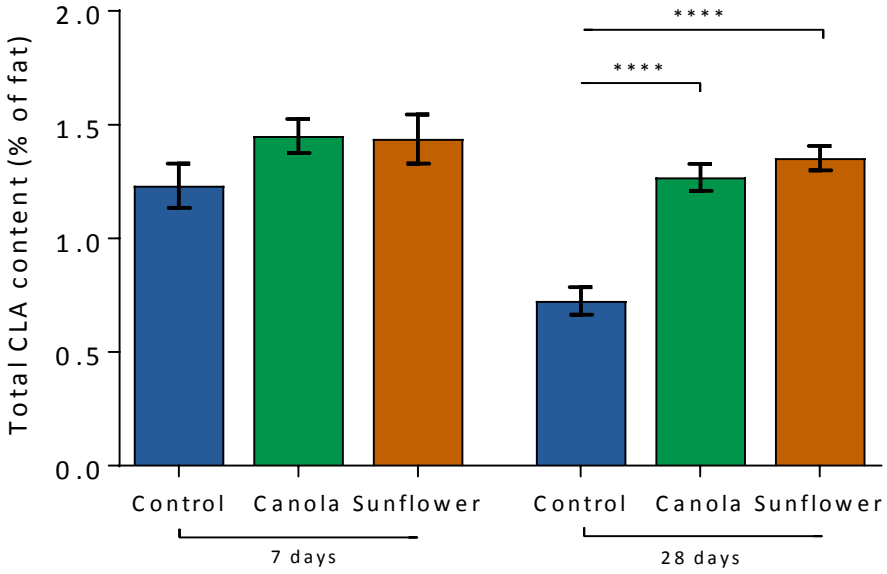

Figure 4.7 Milk total conjugated linoleic acid (CLA) content (% of fat) at 7 and 28 days of lactation.

Ewes fed a control diet (control; day 7 n=7, day 28 n=8), a control diet + 3% canola oil (canola; day 7 n=6, day 28 n=8) or a control diet + 3% sunflower oil (sunflower; day 7 n=6, day 28 n=7). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by ****p<0.0001.

4.5.1.7 *Cis*-9, *trans*-11 CLA isomer

As the major isomer of CLA, the *cis*-9, *trans*-11 content of milk followed a similar pattern to that of total CLA. At day 7 there was no effect of dietary intervention on *cis*-9, *trans*-11 milk concentrations. By day 28 there was 73% ($p<0.0001$) more *cis*-9, *trans*-11 CLA in the milk of ewes supplemented with canola oil and 86% ($p<0.0001$) more in those supplemented with sunflower oil, as shown in Figure 4.8. As with total CLA, it is apparent that rather than causing an increase in the milk *cis*-9, *trans*-11 content, the addition of 3% canola/sunflower oil to the ewe's diet prevents the decrease between 7 and 28 days, which is observed in controls.

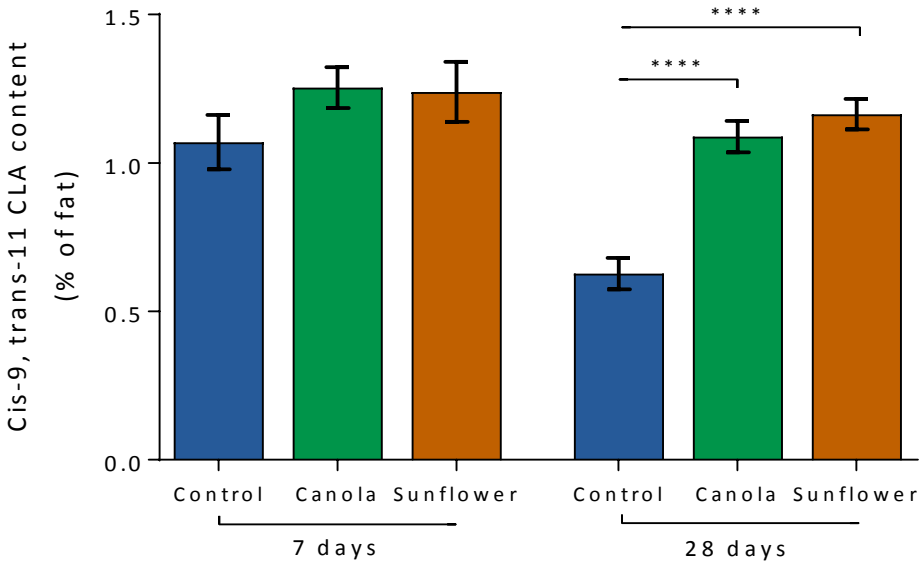

Figure 4.8 Milk *cis*-9, *trans*-11 conjugated linoleic acid (CLA) content (% of fat) at 7 and 28 days of lactation.

Ewes fed a control diet (control; day 7 n=7, day 28 n=8), a control diet + 3% canola oil (canola; day 7 n=6, day 28 n=8) or a control diet + 3% sunflower oil (sunflower; day 7 n=6, day 28 n=7). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by ****p<0.0001.

4.5.1.8 *Trans*-10, *cis*-12 CLA isomer

No effect of maternal supplementation on the *trans*-10, *cis*-12 CLA content of milk was seen at day 7. However, the sunflower group showed a significant increase of 177% ($p=0.0005$) at day 28, compared with controls (Figure 4.9).

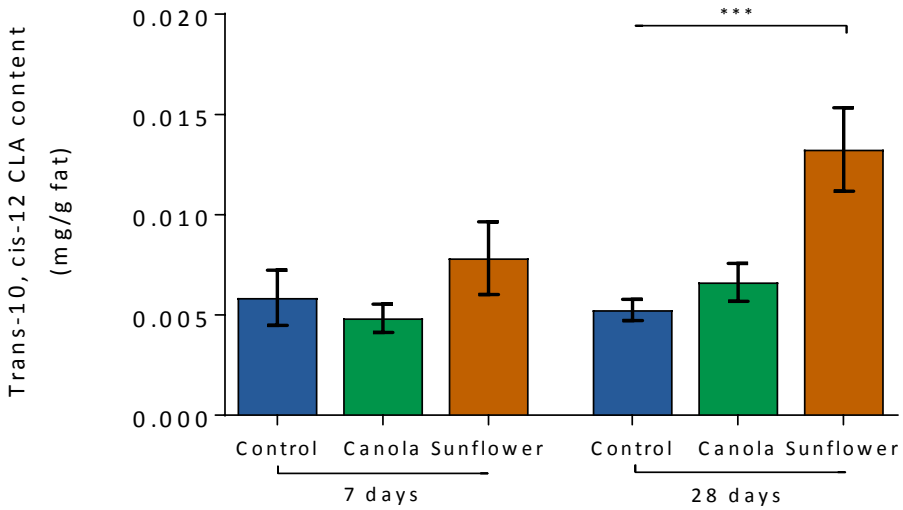

Figure 4.9 Milk *trans*-10, *cis*-12 conjugated linoleic acid (CLA) content (% of fat) at 7 and 28 days of lactation.

Ewes fed a control diet (control; day 7 n=7, day 28 n=8), a control diet + 3% canola oil (canola; day 7 n=6, day 28 n=8) or a control diet + 3% sunflower oil (sunflower; day 7 n=6, day 28 n=7). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by *** p=0.0005.

4.5.1.9 Other CLA isomers

Concentrations of a range of other CLA isomers were measured in the milk samples at both 7 and 28 days. Results are tabulated in Table 4.6 and Table 4.7.

Generally, supplementation of canola or sunflower oil increased the concentrations of CLA isomers present in the milk, especially at 28 days. The CLA content of the milk of supplemented ewes was generally higher than controls at 7 days, but most results were not statistically significant (shown in Table 4.6). By 28 days were clear statistically significant effects of supplementation, particularly with sunflower oil (shown in Table 4.7).

Isomer	Control (n=7)	Canola (n=6)	Sunflower (n=6)	Effect of supplement (% change)
***trans*-7, *cis*-9**	0.029±0.002	0.044±0.003**	**** 0.047±0.002	52%↑ p=0.001 62%↑ p<0.0001
***trans*-12, *trans*-14**	0.012±0.002	0.015±0.002	0.014±0.001	↔ p=0.21 ↔ p=0.19
***trans*-11, *trans*-13**	0.019±0.002	0.026±0.003	0.021±0.001	↔ p=0.06 ↔ p=0.41
***trans*-10, *trans*-12**	0.0027±0.003	0.0028±0.0003	0.0063±0.001*	↔ p=0.71 133%↑ p=0.003
***trans*-9, *trans*-11**	0.015±0.001	0.015±0.001	0.018±0.001	↔ p=0.84 ↔ p=0.06
***trans*-8, *trans*-10**	0.002±0.0003	0.0017±0.0002	0.0026±0.0003	↔ p=0.35 ↔ p=0.18
***trans*-7, *trans*-9**	0.0036±0.0004	0.0043±0.001	0.0048±0.001	↔ p=0.40 ↔ p=0.053
***cis*-12, *trans*-14**	0.0063±0.01	0.0066±0.01	0.0066±0.01	↔ p=0.82 ↔ p=0.84
***trans*-11, *cis*-13**	0.044±0.004	0.055±0.003*	0.048±0.006	25%↑ p=0.036 ↔ p=0.54
***trans*-8, *cis*-10**	0.022±0.002	0.0201±0.001	0.0199±0.002	↔ p=0.41 ↔ p=0.43

Table 4.6 Milk content of other conjugated linoleic acid (CLA) isomers (% of fat) at 7 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Within sampling day, the mean of each treatment group was compared with corresponding controls, by an unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

Isomer	Control (n=8)	Canola (n=8)	Sunflower (n=7)	Effect of supplement (% change)
***trans*-7, *cis*-9**	0.022±0.002	0.063±0.005****	0.064±0.004****	186%↑ p<0.0001 191%↑ p<0.0001
***trans*-12, *trans*-14**	0.005±0.001	0.011±0.001***	0.008±0.0007*	120%↑ p=0.0008 60%↑ p=0.002
***trans*-11, *trans*-13**	0.010±0.002	0.020±0.001***	0.015±0.002*	100%↑ p=0.0003 50%↑ p=0.036
***trans*-10, *trans*-12**	0.0022±0.0002	0.0047±0.0006**	0.009±0.001****	114%↑ p=0.0016 309%↑ p<0.0001
***trans*-9, *trans*-11**	0.013±0.0008	0.017±0.001*	0.021±0.002***	31%↑ p=0.012 62%↑ p=0.0001
***trans*-8, *trans*-10**	0.0022±0.0004	0.0020±0.0002	0.0048±0.0006**	↔ p=0.59 118%↑ p=0.002
***trans*-7, *trans*-9**	0.004±0.0004	0.005±0.0004	0.006±0.0003***	↔ p=0.21 50%↑ p=0.0009
***cis*-12, *trans*-14**	0.0033±0.0004	0.0042±0.0004	0.0048±0.0002**	↔ p=0.12 45%↑ p=0.0062
***trans*-11, *cis*-13**	0.015±0.002	0.025±0.001**	0.020±0.001	67%↑ p=0.0015 ↔ p=0.11
***trans*-8, *cis*-10**	0.017±0.002	0.021±0.001	0.022±0.001*	↔ p=0.11 29%↑ p=0.026

Table 4.7 Milk content of other conjugated linoleic acid (CLA) isomers (% of fat) at 28 days of lactation.

Samples from ewes fed a standard diet (control), a standard diet + 3% canola oil (canola), or a control diet + 3% sunflower oil (sunflower). Values are mean ± SEM. Within sampling day, the mean of each treatment group was compared with corresponding controls, by an unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05) in colours corresponding to treatment group (green=canola; orange=sunflower).

4.5.2 Ewe body weight

Ewes were weighed on days 7 and 28. As shown in Figure 4.10, there were no significant effects of the diet on ewe body weights at either day 7 or day 28.

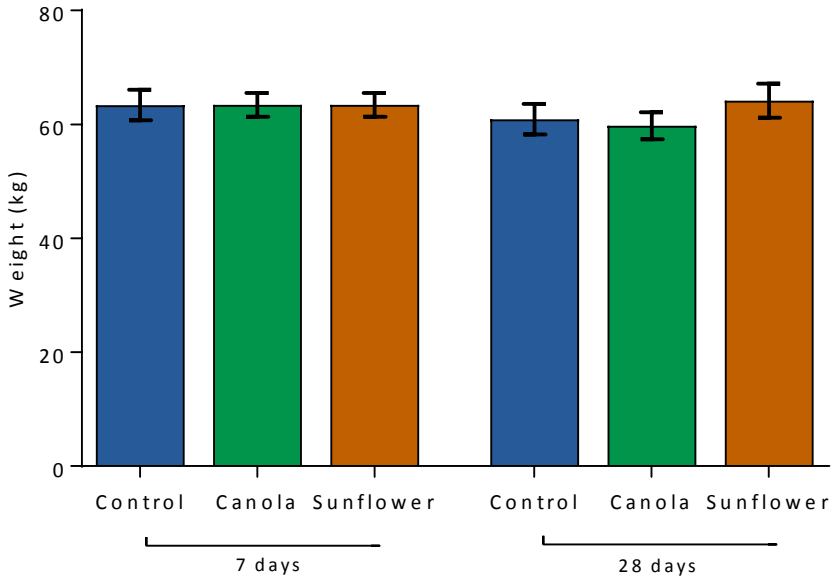

Figure 4.10 Ewe body weights (kg), by dietary group at 7 and 28 days.

Dietary group relates to ewes diet, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Day 7: n=9 per group. Day 28: Control, n=10; canola, n=8; sunflower, n=9. Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. There were no significant differences.

4.6 Discussion

4.6.1 Milk CLA content was increased with canola and sunflower oil supplementation

There are two main ways in which CLA is incorporated into milk fat, 1) from the rumen, where it is derived from LA, 2) from vaccenic acid, by endogenous synthesis via *delta*-9 desaturase in the mammary gland[491]. The slightly larger increase in total CLA and the *cis*-9, *trans*-11 isomer seen in the sunflower group is likely to be due to the higher LA content of sunflower oil compared with canola oil[482]. However, the relatively small difference indicates that the majority of CLA is not derived from the bioconversion of LA in the rumen but is either a result of utilisation of vaccenic acid, or is from endogenous synthesis by Δ^9 desaturase in the mammary gland[491].

My study measured the concentrations of 12 isomers of CLA, as well as total CLA. For many of the isomers, at the time of writing, no biological functions have been attributed, yet my study has shown that dietary supplementation of 3% sunflower oil or 3% canola oil can increase a range of CLA isomers in the milk.

My focus will be on the two main biologically active isomers: the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers.

I have shown that the addition of canola and sunflower oils to the diet of ewes both increased both total CLA and the *cis*-9, *trans*-11 isomer present in milk at 28 days of lactation. Milk CLA content in ruminants varies between seasons due to changes in diet, with fresh grass in summer months causing an increase in milk CLA[363]. The extent to which *cis*-9*, trans*-11 is increased in the summer varies between studies, with increases of 42-183% from winter to summer being observed in cows[361,492]. This variability is likely to be caused by a combination of factors, including breed[493,494] and the nutritional value of the grass. A study in sheep found a smaller increase of 34% in summer compared with winter[363]. The increases in *cis*-9, *trans*-11 seen with supplementation in my study fall somewhere in the middle of those reported with seasonal variation, with a 73% increase at 28 days with canola oil supplementation and an 86% increase with sunflower oil. This suggests that these supplements could be given during winter months to maintain milk CLA content all year round.

4.6.2 Sunflower oil supplementation increases milk *trans*-10, *cis*-12 CLA

In the sunflower oil group, I saw a large increase of 177% in the *trans*-10, *cis*-12 isomer concentrations in milk at 28 days. This effect was not seen with canola oil supplementation. This increase is not necessarily favourable in milk for human consumption, as several negative health effects are connected with this isomer, including decreasing the HDL:LDL cholesterol ratio in adult humans[308]. Effects of the *trans*-10, *cis*-12 isomer on humans in early life are not well known, but its presence in human breast milk is low (approximately 0-5% of CLA)[368,374], whereas the *cis*-9, *trans*-11 isomer naturally makes up the largest proportion of CLA in human breast milk (80-90% of CLA)[366,368] and ruminant milk[495]. In vegetable oils, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers each contribute around 40% of total CLA[341] and commercially available CLA supplements from human consumption generally contain considerably more *trans*-10, *cis*-12 CLA (often around 50%) than that found in ruminant products. Whilst in adult humans, the propensity for the *trans*-10, *cis*-12 isomer to potentially reduce body fat[307,313-318] might be beneficial, this would not be a benefit for infants. Infant body fat plays a key role in the storage of energy as free fatty acids in the form of triglycerides, acting as precursors to ketone bodies which are key for brain lipid synthesis[496] and as a storage site for essential LCPUFAs[497]. Mixed isomer supplements may have a detrimental effect on growth rates in young mice[314]. There is also evidence suggesting that the *trans*-10, *cis*-12 isomer may have a detrimental effect on blood lipid profile whereas the *cis*-9, *trans*-11 isomer has been shown to have a beneficial effect[308].

4.6.3 Medium-chain saturated fatty acids are decreased with canola and, to a lesser extent, sunflower oil supplementation

Overall, I observed a decrease in milk medium-chain SFAs with canola or sunflower oil supplementation and an increase in the longer chain SFAs. The canola oil supplementation induced a larger effect than sunflower oil. Several other studies have shown that increased supply of long-chain FAs to the mammary gland (e.g. through increased dietary long-chain fatty acids) has an effect on medium-chain SFAs in the milk[498,499]. SFA intake in adults is usually associated with an increased risk of negative health outcomes in adults, such as CVD and the metabolic syndrome[500]. However, some research suggests that SFAs should be considered individually[501] with regard to their effects on health, as there are some exceptions.

With regard to supplementation decreasing palmitic acid (C16:0), the WHO suggested in 2003 that there was convincing evidence that palmitic acid increased the risk of developing CVD[502], yet in adults , palmitic acid is more favourable than some other SFAs as it has been shown to induce an 11% decrease in LDL cholesterol when consumed in place of lauric (C12:0) and myristic acid(C14:0)[503]. However, these findings cannot be extrapolated to infants. Palmitic acid contributes to around 20% of human breast milk[504,505], which equates to approximately 10% of total energy and constitutes approximately 50% of the fatty acids present in an infant's adipose tissue[281,506-509]. The human mammary gland has evolved complex pathways in order to synthesise fatty acids and triglycerides, and it is an evolutionary assumption that this is to benefit the infant and/or mother[504,510].

Stearic acid (C18:0), which is a long-chain fatty acid, is considered to be different to other SFAs as it has been found to either not affect[511,512] or decrease[501,513] LDL cholesterol in human blood. Any possible effects in early life are unknown. The increase in stearic acid I observed with supplementation is likely to be due to the high proportion of dietary C18 unsaturated fatty acids which the supplements contain. These are hydrogenated and converted to stearic acid, the end point of C18:0 PUFA biohydrogenation[481].

The decreases in lauric acid (C12:0) and myristic acid (C14:0) which I observed are particularly favourable, due to research showing they exhibit potential negative effects on serum cholesterol and lipoprotein concentrations in adults[503,514]. No specific research has been conducted in early life, but due to the mentioned effects in adults, Koletzko *et al.*[515] have recommended that the sum of myristic acid and lauric acid should not exceed 20% of total fat in infant formula.

4.6.4 Canola and sunflower oil supplementation during lactation increases milk MUFAs

There was a significant increase in MUFA levels in both the canola and sunflower group, compared to controls at 28 days, but only in the canola group at 7 days. Canola oil contains approximately 64% MUFAs, whilst sunflower oil has much lower levels of around 16%[516]. As canola oil has a much higher proportion of MUFAs than sunflower oil[482], the similar concentrations seen with both supplementary fatty acids at 28 days suggest the main cause of this increase is not dietary MUFA intake. It is possible that fatty acid supplementation decreased the rate of ruminal biohydrogenation, which would result in an increase in MUFAs. In cows, supplementing unsaturated fatty acids decreases ruminal biohydrogenation of oleic acid (C18:1)[517]. In my study, milk concentrations of oleic acid

were increased in the canola and sunflower group's milk, suggesting that the biohydrogenation of oleic acid was decreased with supplementation. Oleic acid is the most abundant MUFA present in milk[518] (accounting for >95% of MUFAs in my study) and can be derived from stearic acid, via *delta*-9 desaturation in the mammary gland[483].

The increase in MUFAs with canola oil supplementation has been seen in other studies. A study in goats found that milk MUFA levels increased with 4 and 6%, but not 2% canola oil supplementation[406]. Another study found an increase in MUFAs when supplementing sunflower or rapeseed oil[408].

4.6.5 Canola oil supplementation causes an increase in erucic acid

The canola, but not sunflower, supplemented groups showed a 72% increase in milk erucic acid (C22:1 n-9) at 28 days. Canola oil is a low erucic acid (0.3-0.6% of fat[519]) derivative of rapeseed oil, which naturally has a high percentage (around 50%) of erucic acid. It was developed because erucic acid has been associated with myocardial alterations in rats[520] and has no known nutritional benefits to humans, therefore Koletzko *et al.* state that it should not exceed 1% of fat in infant formula[515]. Despite levels being raised in the canola supplemented group, percentages were all still well within this limit. It is likely that the erucic acid contained in the canola oil supplementation[521] is responsible for the increase in milk erucic acid. This is because long-chain fatty acids are usually derived from dietary sources, rather than *de novo* synthesis[181,182].

4.6.6 Canola oil supplementation decreases omega-6 fatty acids and sunflower oil increases omega-6 linoleic acid

Whilst other effects on milk fatty acids induced by canola and sunflower oil supplementation have been in a similar direction, it appears their effects on milk omega-6 fatty acids differ. In general omega-6 fatty acids were decreased with canola oil supplementation, although levels of the most abundant omega-6 fatty acid, LA (C18:2) remained the same. In contrast, the majority of omega-6 fatty acids were unaffected by sunflower oil supplementation, but LA (C18:2) levels increased. The significant increase in LA content in the milk of sunflower supplemented ewes could increase the adiposity of offspring, as LA increases adiposity in early life in rodents[286], and *in vitro* LA and its derivative AA appear to promote differentiation of preadipocytes, increase fat mass, and increase adipocyte number and size[282,289,522,523].

4.6.7 Canola and, to a greater extent, sunflower oil supplementation increases milk *trans* fatty acids

I found an increase in TFAs in both supplemented groups at 7 and 28 days, with a larger increase in the milk of sunflower supplemented ewes. Part of the increase of TFAs seen in the supplemented ewes' milk can be explained by the increase in CLA, which is classed as a TFA.

Despite the large increase demonstrated here, the absolute TFA values in my study are still far lower than those reported in sheep milk in other studies[363,524,525]. The highest mean milk TFA content in my study was 1.06% of total fatty acids, in the sunflower group at 28 days. Talpur *et al.* reported minimum TFA content of 2.59% and a maximum of 3.59% of total fatty acids in sheep milk[363]. The differences from values demonstrated here may be due to the breed of sheep. My study used Bluefaced Leicester cross Swaledale mule ewes which are a cross of two British breeds, whereas Talpur *et al.* used Kooka sheep, which are native to Pakistan (where the study was conducted) and genetic differences have been shown to affect the milk fatty acid profile of sheep[469].

In early life, TFAs may have a negative impact on essential fatty acid chain elongation, lipoprotein metabolism and growth[526-528]. For this reason, The EFSA has set an upper limit of 3% of total fat for TFAs in infant formula[391]. All of the TFA concentrations measured in my study, regardless of dietary group, still fall well within this limit, with the highest levels of around 1% of fat being observed in the sunflower group.

4.6.8 Total fat content of milk is unaffected by supplementation

There was no effect of either canola or sunflower oil supplementation on total levels of milk fat at either 7 or 28 days. Previous studies investigating the effect of diet on milk fat content have yielded mixed results, suggesting that other factors may be more influential. In cows, Lock *et al.* report that the lowest milk fat concentrations were in December/February (when cows consume little fresh grass) and the highest in May and October (when cows are out to pasture)[361], whereas Heck *et al.* found levels of cows' milk fat to be lowest in grass feeding months[360]. Carta *et al.* reported that seasonal effects of fat content on milk were irregular[529]. In other studies comparable to mine that supplemented lactating ruminants, DePeters *et al.*[456] found that supplementing the diet of cows with 1.6% canola oil did not have a marked effect on milk fat yield, whilst Jenkins *et al.*[455] reported a decrease in milk fat when supplementing cows with 3.5% canola oil. It seems likely that the

effects are dependent on the dose administered. To my knowledge, no studies have supplemented sheep with canola oil and measured milk fatty acids. A study by Mir *et al.*[410] supplemented goats with 2%, 4% and 6% canola oil and found a linear increase in milk fat percentage with increased concentrations of canola oil supplements.

Toral *et al*. found that supplementing ewes diets with 2% sunflower oil had no effect on milk fat levels[530] and neither did a subsequent study by the same group, using a 2.5% sunflower oil supplement[531]. However, Hervás *et al.* found that supplementing sheep with 6% sunflower oil results in an increase in milk fat[409]. As with canola oil, this suggests that the effects are dose-related.

4.6.9 No effects of either supplement were seen on omega-3 fatty acids

Milk EPA and DHA levels are of particular interest due to their potential beneficial effects on human health. In adults, EPA/DHA supplementation appears to reduce the risk of cardiovascular deaths[532] and in infants, DHA is vital for brain development[533]. They also have potential for obesity prevention by activating *UCP1*[191], a key mediator of adaptive thermogenesis which expends energy[76]. My study found no effect of either supplement on any of the omega-3 fatty acids at either time point. As ruminant diets are generally very low in, or void of, EPA and DHA, ruminant products tend to contain very little of these fatty acids. Dietary supplementation of canola combined with fish oils to ruminant animals are effective in increasing omega-3 fatty acids[479]. A study by Welter *et al.* successfully increased milk omega-3 fatty acid content by supplementing 6% but not 3% canola oil[407]. Therefore, it is possible that by increasing the dose we may be able to increase milk omega-3 fatty acids, however, addition of marine oils/algae to our existing supplements is likely to be more effective[534,535].

4.6.10 Comparison with Study A (Chapter 3)

This study (Study B) was successful in altering milk fatty acid profile by the supplementation of canola or sunflower oil. Canola and sunflower oil supplementation led to a decrease in medium-chain SFAs and an increase in MUFAs and CLA. However, Study A (Chapter 3) was largely unsuccessful in altering the milk composition, showing no effect of canola oil supplementation on the majority of tested SFAs, MUFAs or the main biologically active isomers of CLA. This is despite both studies being carried out with sheep, using identical control and supplemented diets, at the same farm, and at a similar time of year.

It is known that ruminant milk fatty acid content can be affected by diet[483]. This study (Study B) and several other studies have successfully altered milk composition by canola or sunflower oil supplementation[406,408,409]. There are a number of potential reasons for the variation in the results seen with canola supplementation between my two investigations. Firstly, as different breeds of sheep were utilised in Studies A and B, this is likely to have been, at least in part, responsible for the variation. This is supported by variations between the controls in Studies A and B, and by other studies, with research in cows and sheep showing that breed can have a significant effect on milk composition[469,493], including CLA concentrations[469,494].

Another factor which may affect the milk fatty acid profile, and more specifically milk CLA content, is parity[470,474]. However, this research is by no means conclusive. Nogalski *et al*. found that primiparous cows have a more favourable milk fatty acid profile than cows in their second and third lactation[470]. They also found a 10% increase in milk CLA levels of primiparous cows. However, in contrast Stanton *et al*. found an increase in milk CLA with lactation number in grass-fed cows[474]. Interestingly, in the same paper, they found no effect of lactation number with cows fed a rapeseed supplement, which is similar to the canola oil supplement fed in this study. CLA is synthesised endogenously in ruminants[536], so potentially endogenous synthesis becomes less effective with parity. Therefore a dietary supplement, such as canola oil, may not be able to be fully utilised and its component fatty acids converted to CLA, by ewes of a higher parity. The sheep used in Studies A and B were known to be multiparous, but the exact number of previous births/lactations is not known. Potentially, if the ewes used in Study A had lambed more/fewer times than those in Study B, this could explain the lesser effect of supplementation in this study.

In both studies pre-study nutritional information was not available, so another potential explanation for the variation is that the ewes may have been fed different diets before the study began. As we know that milk CLA content is strongly influenced by diet[360,361,363], this could be of potential importance. Study B has shown that when canola oil is supplemented from the day of birth, no significant increase in CLA is observed at 7 days, but there is a significant increase at 28 days. Other studies have found a much faster effect of canola oil[410], seeing increases in milk CLA from 2 days of supplementation, however, this was in late lactation. To my knowledge, there are no studies investigating the effect of supplementation on CLA at the start of lactation. This may suggest that the synthesis of CLA is slower in the first weeks of lactation, therefore pre-study diet may still influence milk composition. If this study were to be repeated, all ewes should be fed a standardised diet

for a set amount of time before delivery, to ensure pre-study diet had minimal effects and ewe weight should be considered as a variable on randomisation.

4.7 Conclusions

For this chapter I hypothesised that canola and sunflower supplementation would decrease SFAs, increase MUFAs, and increase total and *cis*-9, *trans*-11 CLA in the milk of ewes.

Both supplements decreased medium-chain (up to C16:0) SFAs, however, canola oil did so to a greater extent than sunflower oil. Both supplements increased milk MUFA concentrations. Canola oil supplementation decreased the majority of omega-6 fatty acids analysed, but not LA, whereas sunflower oil led to an increase in LA and total omega-6 fatty acids. Canola and sunflower supplementation both increased the total and *cis*-9, *trans*-11 CLA concentrations, as hypothesised. However, sunflower oil supplementation also increased concentrations of *trans*-10, *cis*-12 CLA. A summary of the main effects of supplementation on milk fatty acid profile is given in Table 4.8.

	Day 7		Day 28	
	Canola	Sunflower	Canola	Sunflower
Total fat				
	↔	↔	↔	↔
Medium-chain saturated fatty acids				
	↓	↔	↓↓	↓
Long-chain saturated fatty acids				
C14:0	↓	↓	↓	↓
C16:0	↓	↓	↓	↓
C18:0	↑	↔	↑	↑
C20:0	↑	↔	↑↑	↑
C22:0	↔	↑	↔	↑
Monounsaturated fatty acids				
Total MUFAs	↑	↔	↑	↑
C18:1 n-9	↑	↔	↑	↑
C22:1 n-9	↔	↔	↑	↔
Omega-3 fatty acids				
	↔	↔	↔	↔
Omega-6 fatty acids				
n-6 PUFAs	↔	↔	↔	↑
n-6 LC PUFA	↓	↓	↓	↔
C18:2 n-6	↔	↔	↔	↑
C18:3n-6	↔	↔	↓	↔
C20:2n-6	↔	↔	↓	↔
C20:4n-6	↓↓	↓	↓	↔
C22:2n-6	↓	↔	↓↓	↓
C22:4n-6	↔	↔	↓	↔
C22:5n-6	↓	↓	↓	↔
***Trans* fatty acids**				
	↑	↑	↑	↑
Conjugated Linoleic acid				
Total CLA	↔	↔	↑	↑
*c*9, *t*11 CLA	↔	↔	↑	↑
*t*10, *c*12 CLA	↔	↔	↔	↑

Table 4.8 Summary of results, showing changes induced in milk fatty acid profile at 7 and 28 days with maternal dietary supplements of either 3% canola or 3% sunflower oil.

Symbols indicate the difference in content compared with controls: ↔=no change, ↑=increase and ↓=decrease. When two symbols are shown (↑↑ or ↓↓) it means the percentage change between that group and controls was largest compared with the other supplemented group at that time point. MUFA = monounsaturated fatty acid. PUFA = polyunsaturated fatty acid.

5 The effect of altered milk fatty acid profile on the body composition, plasma metabolites and thermogenic capacity of adipose tissue in lambs

5.1 Introduction

The first part of 'Study B' was detailed in Chapter 4 and outlined the effects which supplementing ewes with either 3% canola or sunflower oil had on the fatty acid profile of their milk. A summary of the changes seen in the milk fatty acid profile, detailed in Chapter 4 can be found in Table 4.8 (Page 162).

The focus of this Chapter is investigating the second part of 'Study B', which is the effect that the altered milk fatty acid profile has had on the offspring, with a specific interest in their adiposity and BAT function.

Chapter 1 has summarised the importance of BAT and its potential to be utilised in order to help prevent and potentially treat obesity[537]. The most common genetic marker of BAT is *UCP1*[84]. This is the protein which uncouples cellular respiration from ATP synthesis resulting in the dissipation of energy as heat. *In vitro* and *in vivo* animal studies suggest that dietary manipulation, but more specifically fatty acids such as CLA[332,334], may increase UCP1. The exact mechanisms by which this occurs are unknown, although one study found that CLA increases noradrenaline[538], which is known to increase UCP1[539]. In addition to classical BAT and WAT, a third type of adipocyte has been identified and termed "brite" (brown in white) or 'beige'. Brite adipocytes are 'brown like' cells within WAT which express *UCP1* and can be induced by cold exposure in mice[123] or by β_3 adrenergic receptor[540] (transcribed by the *ADRB3* gene) and *PPARγ*[541] agonist drugs. These cells seem to be from a different lineage to classical BAT[137]. Nonetheless, when activated they have a similar thermogenic capacity to classical BAT[129] and are therefore, also attractive to researchers as a potential target for the treatment of obesity.

Current research into CLA and BAT is generally limited by two main factors. Firstly, the majority has been conducted *in vitro*, or in rodents. To my knowledge, no studies on CLA and BAT have been carried out in larger mammals. The other limitation is that CLA occurs in numerous isomers and the two main biologically active isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) appear to have different/opposing effects on blood lipids[308], body weight[542] and milk fat content[382] and therefore it is possible that this is also the case for their effects on BAT. This leads to difficulty when interpreting and comparing studies, as it is not clear

whether differing results are caused by the variable ratio of isomers administered. As shown in Chapter 4, (Table 4.8, Page 162), maternal canola oil supplementation increased the milk content of the *cis*-9, *trans*-11 isomer but did not alter concentrations of the *trans*-10, *cis*-12 isomer, whereas, maternal sunflower oil supplementation increased concentrations of both isomers.

Whilst results can be attributed to maternal canola/sunflower oil supplementation, it is not possible to establish exactly which fatty acids are responsible for effects on the offspring. This is due to numerous aspects of the milk fatty acid profile changing, however, this study will help determine whether the fatty acid composition of the diet in early life can affect BAT function.

Expression of mRNA for a selection of genes associated with brown, white and brite adipose tissue, adipogenesis and metabolism was measured, in order to assess the effects that different milk fatty acid profiles may have on the offspring. The definitive marker of BAT, UCP1, was investigated in more detail using immunohistochemistry and Western blotting to detect the abundance of the protein. All gene analysis was conducted on the perirenal adipose tissue depot. This depot was the largest depot and previous studies have confirmed that it is BAT at birth, transitioning to WAT by 28 days[100]. This allowed me to use this critical window to investigate whether this decrease can be reduced or prevented. The lambs' plasma metabolites were also assessed in order to get a more complete view of the effects of maternal supplementation.

NEFAs, triglycerides and total cholesterol were tested to establish if the altered milk fatty acid profile had an effect on blood lipids. Plasma IGF-1 and leptin were analysed due to their association with fat mass[543]. Plasma glucose was measured to give an indication of whether altered milk composition had any effect on glucose control. Urea was measured as it had been found to be elevated in lambs fed a high fat diet[544] and because it indicates kidney function[545]. Plasma gamma-glutamyl transpeptidase (GGT) was measured to give an indication of liver function, as in Study A (Chapter 3) a small effect of maternal canola supplementation was seen on female offspring's liver weights. Finally, plasma acetate was investigated due to it being linked to upregulating *UCP1* expression in rodents[439].

5.2 Hypotheses

As detailed in Chapter 4 and summarised in Table 4.8 (Page 162), total and *cis*-9, *trans*-11 CLA increased in the milk of both canola and sunflower oil supplemented ewes. Due to research suggesting CLA may increase UCP1[332,334], I hypothesised that I would see an increase in UCP1 in lambs fed by these ewes. Research is conflicting with regard to the effect of specific CLA isomers[330,332], therefore I hypothesised that the increase in the *trans*-10, *cis*-12 isomer seen in the sunflower group would modulate my hypothesised increase in UCP1.

There were elevated concentrations of linoleic acid (LA, C18:2 n-6) in the milk of sunflower supplemented ewes. LA and its derivative arachidonic acid (AA, C20:4 n-6) have been linked with increased body fat[288,289] and promote the differentiation of pre adipocytes[286]. I therefore hypothesised that the lambs fed by ewes receiving a supplement of sunflower oil in their diet would have increased adipose tissue, body weight and growth rates.

5.3 Methods

A detailed description of the methods used in my investigation can be found in Chapter 2. Figure 5.1 provides an overview of the animal study described in this Chapter.

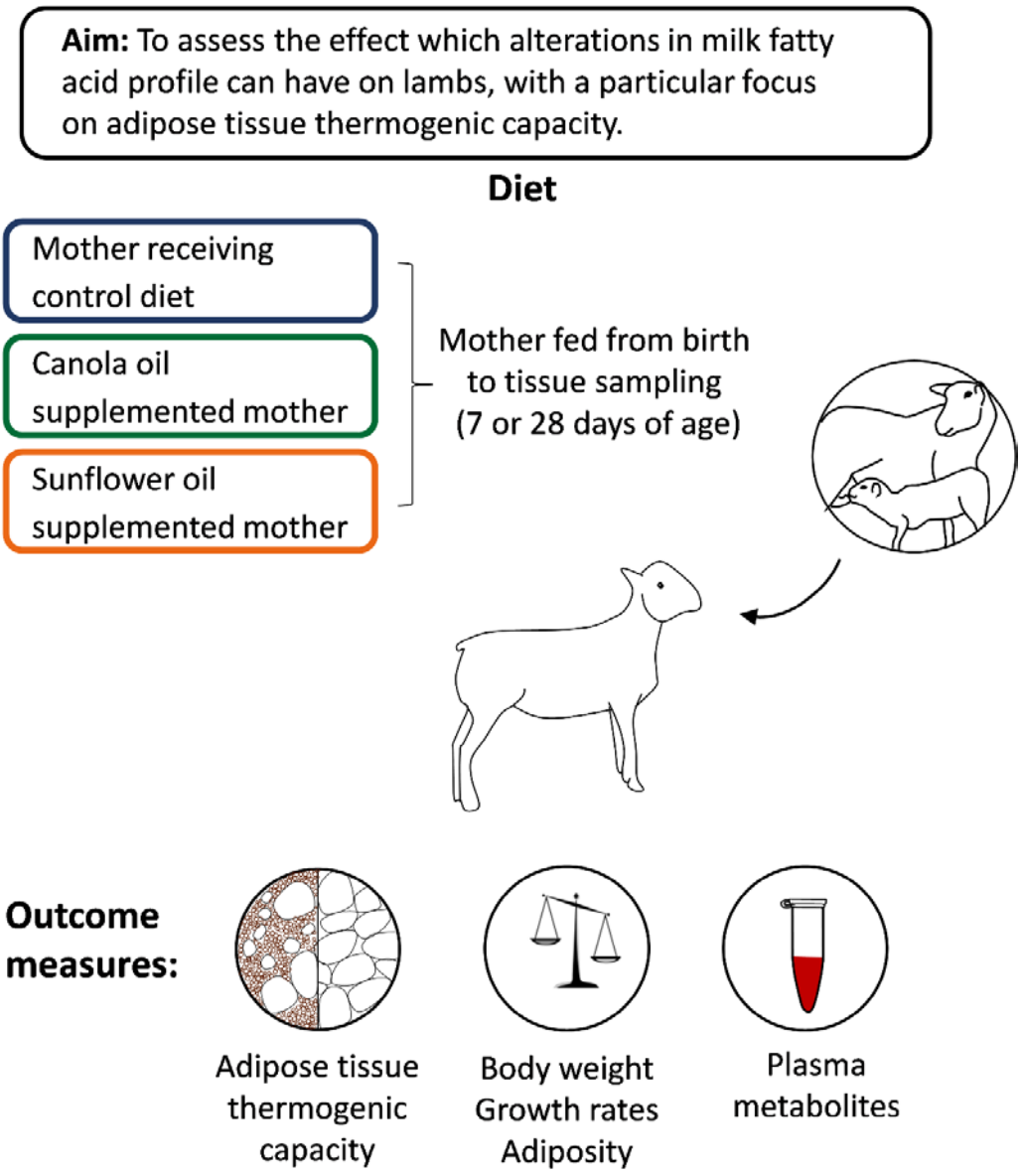

Figure 5.1 Summary of experimental design.

Day numbers refer to days of age.

All reported animal experimentations for this study were performed by Professor Michael Symonds, Dr Mark Birtwistle, Dr Viv Perry and Mr Graeme Davies at the University of Nottingham, Sutton Bonington campus. Leptin and IGF-1 analyses were performed by Professor Duane Keisler at the University of Missouri, USA. All other plasma metabolite work was conducted by myself and Dr Nigel Kendall at the University of Nottingham's

School of Veterinary Medicine, Sutton Bonington Campus. The perirenal adipose tissue for histology was processed and analysed by myself, with assistance from Sarah Simeen. All other gene and protein expression work was performed by myself.

5.4 Results

There were times when data for all lambs was not able to be collected, for technical reasons or due to difficulty sampling. Numbers of lambs per group available for analysis are given in each figure and table.

5.4.1 Body weights

From birth until they were euthanased and their tissues sampled (at either 7 or 28 days of age) lambs were allowed to freely suckle from their mothers. Lamb milk intake was not measured, so it is not known if it was the same between groups. However, there were no significant differences between the groups' birth or body weights at 7 or 28 days (Figure 5.2). Weight gain (kg per day) between birth and 7 days, and birth and 28 days were calculated and female lambs in the sunflower group showed greater weight gain between birth and 7 days (on average 0.35kg/day), compared with 0.26 kg/day in controls ($p=0.03$, Figure 5.3).

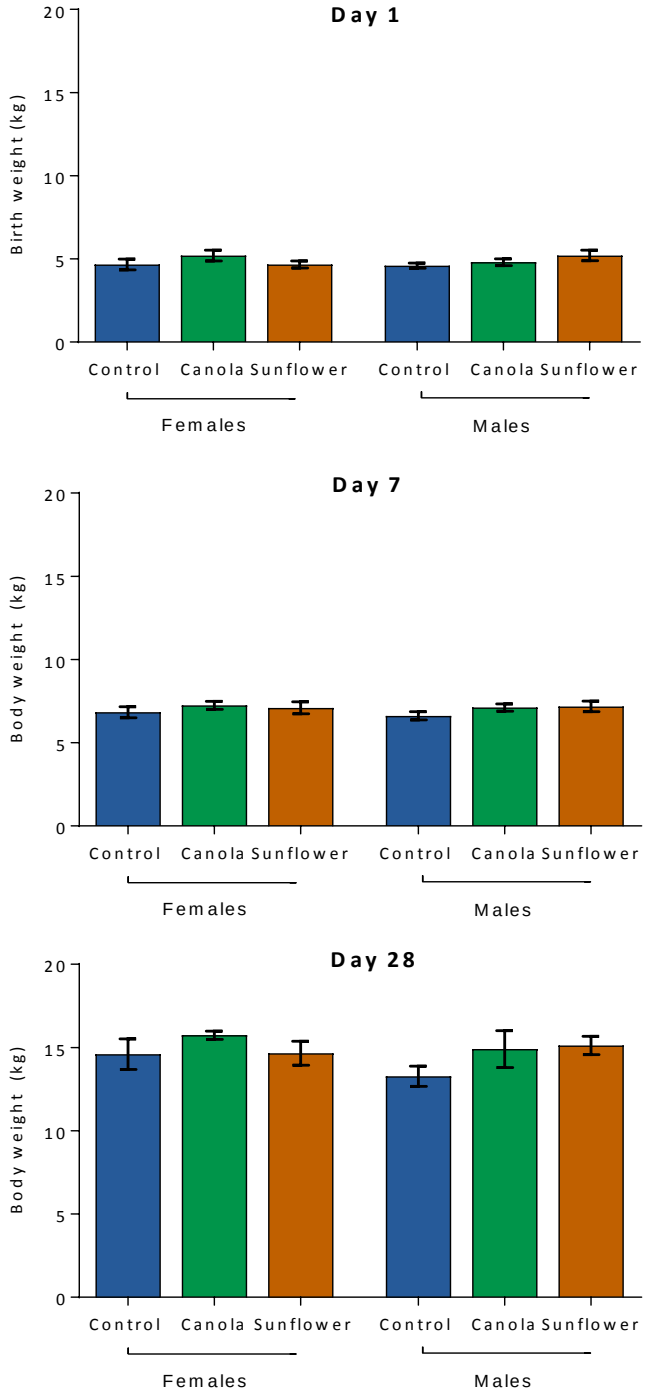

Figure 5.2 Lamb body weights (kg), by dietary group and gender, at 7 and 28 days.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). At 7 days, females: control, n=9; canola, n=8; sunflower, n=10. Males: control, n=7; canola, n=7; sunflower, n=8. At 28 days, females: n=5 per group. Males; n=4 per group). Values are mean ± SEM.

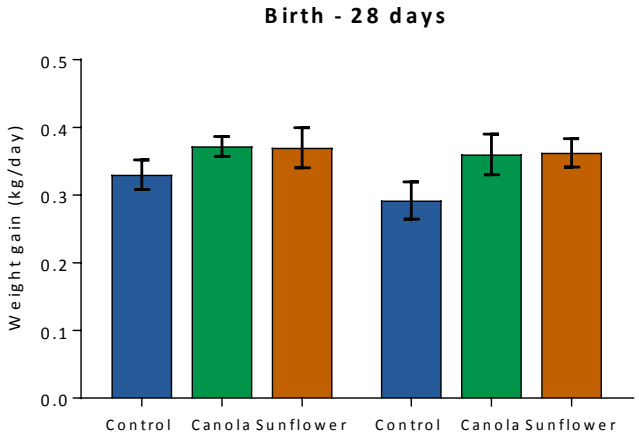

Figure 5.3 Lamb weight gain (kg per day) by dietary group and gender, from birth to 7 and 28 days.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). For birth - 7 days, females: control, n=9; canola, n=8; sunflower, n=10. Males: control, n=7; canola, n=7; sunflower, n=8. For birth - 28 days, females: n=5 per group. Males; n=4 per group). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by * p=0.03.

5.4.2 Organ weights

There were few changes in organ weights in response to maternal dietary supplementation during lactation. Those which were observed were only seen in male lambs. No changes were seen at 7 days (Table 5.1). At 28 days, males in the sunflower group showed a 15% decrease in brain weight (relative to body weight). Male lambs in the canola group showed decreases in heart and lymph node relative weights of 10% and 29% respectively, compared with controls. Results are shown in Table 5.2.

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	Female				Male			
Organ	Control (n=4)	Canola (n=5)	Sunflower (n=5)	p value	Control (n=4)	Canola (n=3)	Sunflower (n=4)	p value
Adrenal	0.083±0.007	0.078±0.012	0.092±0.010	↔p=0.73 ↔ p=0.47	0.085±0.001	0.082±0.010	0.079±0.007	↔p=0.72 ↔ p=0.41
Brain	7.25±0.36	7.07±0.35	6.48±0.60	↔p=0.74 ↔ p=0.34	7.32±0.26	7.10±0.31	7.18±0.65	↔p=0.60 ↔ p=0.85
Heart	7.70±0.29	7.69±0.32	8.21±0.47	↔p=0.98 ↔ p=0.42	7.58±0.13	7.53±0.52	7.77±0.30	↔p=0.92 ↔ p=0.57
Kidneys	6.99±0.30	6.39±0.40	6.26±0.24	↔p=0.28 ↔ p=0.10	6.99±0.10	6.87±0.35	6.89±0.72	↔p=0.71 ↔ p=0.90
Liver	25.45±1.69	23.00±1.13	25.76±0.83	↔p=0.27 ↔ p=0.87	27.65±0.50	27.23±0.77	25.73±1.13	↔p=0.63 ↔ p=0.16
Lungs	23.15±0.81	26.35±2.34	20.48±1.03	↔p=0.25 ↔ p=0.10	21.40±1.14	23.37±1.79	21.93±0.37	↔p=0.38 ↔ p=0.68
Lymph node	0.51±0.08	0.49±0.05	0.45±0.05	↔p=0.90 ↔ p=0.55	0.57±0.04	0.49±0.02	0.50±0.09	↔p=0.19 ↔ p=0.87
Pancreas	0.72±0.04	0.67±0.05	0.81±0.18	↔p=0.47 ↔ p=0.68	0.67±0.10	0.74±0.05	0.89±0.04	↔p=0.62 ↔ p=0.12
Spleen	2.90±0.41	2.78±0.31	2.67±0.44	↔p=0.83 ↔ p=0.72	3.16±0.21	3.45±0.46	1.94±0.96	↔p=0.56 ↔ p=0.26

Table 5.1 Lamb organ weights, relative to body weight (g/kg) at 7 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

Organ	Female			Effect of supplement (% change)	Male			Effect of supplement (% change)
	Control (n=5)	Canola (n=5)	Sunflower (n=5)		Control (n=4)	Canola (n=4)	Sunflower (n=4)	
Adrenal	0.057±0.005	0.055±0.004	0.058±0.004	↔p=0.64 ↔ p=0.95	0.052±0.014	0.054±0.002	0.058±0.011	↔p=0.90 ↔ p=0.74
Brain	4.30±0.38	3.93±0.14	4.34±0.26	↔p=0.39 ↔ p=0.93	4.24±0.19	4.21±0.30	3.61±0.17*	↔p=0.92 15% ↓p=0.047
Heart	6.38±0.26	6.53±0.35	6.56±0.18	↔p=0.75 ↔ p=0.57	6.96±0.11	6.23±0.25*	6.86±0.54	10% ↓p=0.037 ↔ p=0.87
Kidneys	5.09±0.20	5.20±0.03	5.41±0.23	↔p=0.63 ↔ p=0.33	5.39±0.15	5.62±0.43	5.34±0.12	↔p=0.62 ↔ p=0.80
Liver	19.82±1.24	22.26±1.22	23.08±0.84	↔p=0.20 ↔ p=0.06	23.58±0.55	22.93±0.90	23.30±1.21	↔p=0.55 ↔ p=0.83
Lungs	18.00±1.24	15.72±0.96	16.96±0.68	↔p=0.19 ↔ p=0.48	16.00±0.49	16.23±0.37	17.75±1.11	↔p=0.73 ↔ p=0.20
Lymph	0.57±0.09	0.48±0.05	0.44±0.04	↔p=0.43 ↔ p=0.24	0.51±0.02	0.36±0.03**	0.44±0.03	29%↓p=0.006 ↔ p=0.10
Pancreas	0.63±0.14	0.63±0.07	0.78±0.18	↔p=0.98 ↔ p=0.55	0.62±0.07	0.77±0.15	0.62±0.09	↔p=0.42 ↔ p=0.98
Spleen	4.46±0.66	4.55±0.63	3.85±0.64	↔p=0.92 ↔ p=0.52	4.09±0.64	4.37±0.64	4.14±0.51	↔p=0.77 ↔ p=0.95

Table 5.2 Lamb organ weights, relative to body weight (g/kg) at 28 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

5.4.3 Adipose tissue weights

Data for adipose tissue weights at 7 and 28 days is shown in Table 5.3 and Table 5.4 respectively.

All tissue and organ weights are shown relative to body weight (grams per kilogram of body weight). The study design dictated that weights and/or sampling would be undertaken on day 1 (birth), day 7 (±1) and day 28 (±3). 'Total' adipose tissue refers to the sum of the adipose tissue depots excised and weighed, these being the omental, pericardial, perirenal and sternal depots.

At day 7 neither female nor male lambs showed any changes in adipose tissue depot weights relative to body weight. At day 28, female lambs in the sunflower group showed significant increases in the omental (17%, p=0.01), pericardial (53%, p=0.03), and perirenal (76%, p=0.0095) adipose tissue depots. The females in the canola group showed a 47% increase in relative sternal adipose tissue weight (p=0.048). No effects were seen in males.

At 7 days there were no effects on 'total' adipose tissue in either group, however, at 28 days females in the canola and sunflower groups had 49% and 29% more 'total' adipose tissue than controls respectively. There were no effects of dietary group in male lambs.

	Female				Male			
Depot	Control (n=4)	Canola (n=5)	Sunflower (n=5)	p value	Control (n=4)	Canola (n=3)	Sunflower (n=4, sternal n=3)	p value
Omental	2.07±0.29	2.18±0.54	1.97±0.57	↔p=0.87 ↔ p=0.89	2.33±0.25	2.28±0.33	1.53±0.28	↔p=0.9 ↔ p=0.07
Pericardial	1.03±0.11	1.09±0.21	1.13±0.10	↔p=0.81 ↔ p=0.53	1.23±0.06	1.34±0.05	1.51±0.52	↔p=0.22 ↔ p=0.61
Perirenal	7.22±0.82	7.70±1.51	8.33±0.92	↔p=0.79 ↔ p=0.41	5.94±0.48	6.93±0.62	5.98±0.68	↔p=0.25 ↔ p=0.96
Sternal	2.60±0.26	2.80±0.89	3.15±0.33	↔p=0.84 ↔ p=0.26	2.16±0.20	2.77±0.67	1.93±0.62	↔p=0.37 ↔ p=0.52
Total	12.9±1.1	13.8±2.9	14.5±1.4	↔p=0.80 ↔ p=0.41	11.7±0.4	13.3±1.5	10.9±1.6	↔p=0.27 ↔ p=0.48

Table 5.3 Lamb adipose tissue depot weights relative to body weight (g/kg) at 7 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

	Female				Male			
Depot	Control (n=5)	Canola (n=5)	Sunflower (n=5)	Effect of supplement (% change)	Control (n=4)	Canola (n=4)	Sunflower (n=4)	Effect of supplement (% change)
Omental	7.29±0.33	8.15±0.43	8.53±0.22*	↔p=0.15 17%↑ p=0.015	8.34±1.60	8.23±0.78	7.83±0.63	↔p=0.95 ↔ p=0.78
Pericardial	1.22±0.10	1.58±0.18	1.87±0.24*	↔p=0.12 53%↑ p=0.034	1.55±0.08	1.65±0.22	1.38±0.14	↔p=0.71 ↔ p=0.34
Perirenal	10.24±2.07	13.58±0.54	18.04±1.03**	↔p=0.15 76%↑p=0.010	8.81±0.88	10.27±1.40	7.42±0.74	↔p=0.42 ↔ p=0.27
Sternal	4.19±0.59	6.16±0.60*	5.82±0.47	47%↑ p=0.048 ↔ p=0.06	5.27±0.91	4.90±0.59	4.86±0.48	↔p=0.74 ↔ p=0.71
Total	22.94±2.58	29.50±0.46*	34.26±0.67**	29%↑p=0.037, 49% ↑ p=0.003	23.98±3.32	25.03±2.74	21.50±0.16	↔p=0.82 ↔ p=0.48

Table 5.4 Lamb adipose tissue depot weights relative to body weight (g/kg) at 28 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

5.4.4 Plasma metabolites

Results for plasma metabolite concentrations at 7 and 28 days are shown in Table 5.5 and Table 5.6 respectively.

There was no effect of dietary group on lambs' plasma acetate, GGT, IGF-1, NEFAs or urea concentrations.

There were no significant effects of dietary group on plasma total cholesterol (mmol/l) concentrations in either gender at 7 days. However, at 28 days there were significant decreases in plasma total cholesterol concentrations female lambs in both the canola (25%, p=0.007) and sunflower (19% p=0.03) groups, when compared to controls. No changes were seen in males at 28 days.

Plasma glucose concentrations were 13% higher in male lambs in the sunflower group at 7 days, compared with controls (p=0.009) but there was no significant difference in females. In contrast, whilst no such effect was seen at 28 days in males, females in the sunflower group showed 16% higher plasma glucose concentrations at 28 days than controls (p=0.003). No changes were seen in the plasma glucose of the canola group lambs at either 7 or 28 days.

At 7 days of age, increases in plasma leptin concentrations were seen in both the canola (26%, p=0.0019) and sunflower (41%, p=0.009) group females, compared with controls.

At 28 days, female lambs in the canola group showed plasma leptin concentrations 80% higher than controls (p=0.03) and in the sunflower group, females plasma leptin concentrations were 62% higher than controls (p=0.03). No effects of dietary group on plasma leptin were observed in male lambs.

Plasma triglycerides in sunflower group females were significantly higher than controls at 7 days (38%, p=0.0096), but by 28 days there were no differences. No effects on triglyceride concentrations were seen in sunflower males or any of the canola group lambs.

	Female				Male			
	Control (n=4)	Canola (n=4)	Sunflower (n=5)	Effect of supplement (% change)	Control (n=4)	Canola (n=3)	Sunflower (n=3)	Effect of supplement (% change)
Acetate (mmol/l)	0.004±0.003	0.01±0.01	0.02±0.01	↔p=0.71 ↔ p=0.87	0.01±0.01	0.02±0.01	0.03±0.002	↔p=0.71 ↔ p=0.27
Total cholesterol (mmol/l)	2.11±0.07	2.19±0.12	1.77±0.12	↔p=0.62 ↔ p=0.06	1.85±0.15	1.71±0.43	2.20±0.24	↔p=0.74 ↔ p=0.24
GGT (I/U)	528±283	403±124	637±159	↔p=0.68 ↔ p=0.73	468±158	622±165	512±148	↔p=0.54 ↔ p=0.85
Glucose (mmol/l)	7.04±0.67	6.67±0.17	6.32±0.42	↔p=0.57 ↔ p=0.37	6.41±0.17	6.03±1.29	7.25±0.04**	↔p=0.74 13% ↑ p=0.009
IGF-1 (ng ml−1)	126±5	100±17	137±16	↔p=0.17 ↔ p=0.57	150±10	169±2	158±9	↔p=0.28 ↔ p=0.63
Leptin (ng ml−1)	2.36±0.08	2.97±0.18*	3.32±0.23**	26%↑ p=0.019 41% ↑ p=0.009	2.79±0.36	2.89±0.32	2.54±0.16	↔p=0.88 ↔ p=0.63
NEFA (mmol/l)	0.24±0.02	0.31±0.09	0.22±0.05	↔p=0.56 ↔ p=0.74	0.30±0.09	0.75±0.24	0.37±0.15	↔p=0.10 ↔ p=0.69
Triglycerides (mmol/l)	0.55±0.02	0.67±0.07	0.76±0.05**	↔p=0.15 38% ↑ p=0.0096	0.51±0.17	0.80±0.30	0.75±0.18	↔p=0.41 ↔ p=0.38
Urea (mmol/l)	2.64±0.91	2.96±0.45	4.34±0.43	↔p=0.75 ↔ p=0.11	3.66±0.43	7.57±3.95	3.08±0.40	↔p=0.30 ↔ p=0.38

Table 5.5 Plasma metabolite values at 7 days, by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

	Female				Male			
	Control (n=5)	Canola (n=5)	Sunflower (n=5)	Effect of supplement (% change)	Control (n=4)	Canola (n=4)	Sunflower (n=4)	Effect of supplement (% change)
Acetate (mmol/l)	0.02±0.005	0.02±0.01	0.03±0.01	↔p=0.66 ↔ p=0.07	0.03±0.003	0.03±0.01	0.03±0.01	↔p=0.79 ↔ p=0.87
Total cholesterol (mmol/l)	5.46±0.32	4.12±0.18**	4.44±0.23*	25% ↓p=0.007 19%↓ p=0.03	4.06±0.42	4.68±0.59	4.00±0.53	↔p=0.42 ↔ p=0.93
GGT (I/U)	90±8	92±8	91±10	↔p=0.88 ↔ p=0.96	122±18	99±9	121±13	↔p=0.30 ↔ p=0.97
Glucose (mmol/l)	6.79±0.17	6.98±0.14	7.88±0.20**	↔p=0.40 16%↑ p=0.003	8.42±0.50	7.72±0.19	7.55±0.37	↔p=0.23 ↔ p=0.21
IGF-1 (ng ml−1)	186±12	228±20	221±31	↔p=0.11 ↔ p=0.31	319±42	254±34	273±9	↔p=0.28 ↔ p=0.33
Leptin (ng ml−1)	2.64±0.20	4.74±0.77*	4.28±0.60*	80%↑ p=0.03 62%↑ p=0.03	3.04±0.42	3.22±0.24	3.24±0.35	↔p=0.72 ↔ p=0.73
NEFA (mmol/l)	0.55±0.10	0.38±0.13	0.47±0.19	↔p=0.32 ↔ p=0.71	0.29±0.11	0.73±0.20	0.48±0.15	↔p=0.10 ↔ p=0.34
Triglycerides (mmol/l)	0.76±0.16	0.71±0.17	0.70±0.07	↔p=0.82 ↔ p=0.75	0.85±0.05	0.83±0.03	1.03±0.28	↔p=0.72 ↔ p=0.56
Urea (mmol/l)	5.40±0.15	5.52±0.55	5.21±0.20	↔p=0.83 ↔ p=0.47	5.37±0.71	4.29±0.29	4.94±0.66	↔p=0.21 ↔ p=0.67

Table 5.6 Plasma metabolite values at 28 days by dietary group and gender.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significant differences are denoted by directional arrows (↑= increase, ↓= decrease, ↔ = no significant change, p>0.05), in colours corresponding to treatment group (green=canola; orange=sunflower).

5.4.4.1 Relationship between plasma leptin and adiposity

Correlations were plotted between the plasma leptin concentrations and the 'total' adipose tissue, which refers to the combined weight of the omental, pericardial, perirenal and sternal adipose tissue excised from the lambs, Figure 5.4. There were no significant correlations between plasma leptin and fat mass.

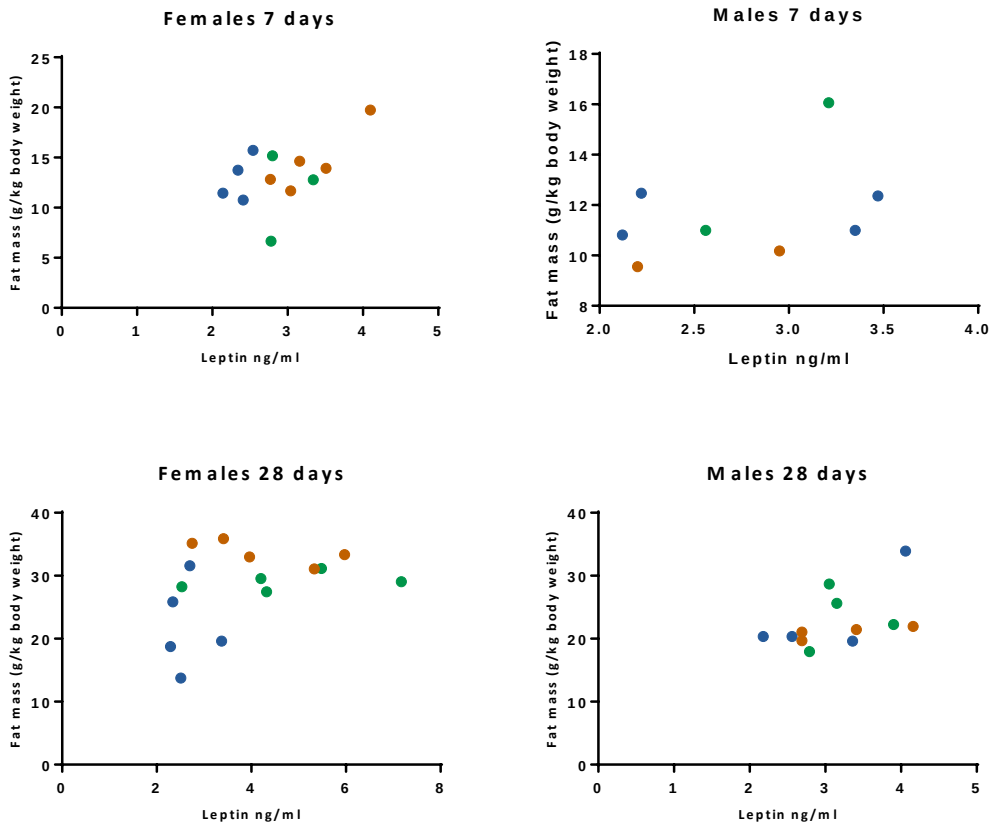

Figure 5.4 Correlations between plasma leptin concentrations (ng/ml) and fat mass (g/kg body weight).

Fat mass refers to the sum of the 4 depots sampled (omental, pericardial, perirenal and sternal). Females 7 days: R^2=0.28, p=0.078. Females 28 days: R^2=0.14, p=0.17. Males 7 days: R^2=0.17, p=0.31. Males 28 days: R^2=0.23, p=0.12. Dietary groups are distinguished by coloured data points. Blue for controls, green for canola and orange for sunflower.

5.4.5 The effect of maternal canola/sunflower oil supplementation on offspring's perirenal adipose tissue gene expression

The mRNA expression of a range of genes was tested in the perirenal adipose tissue of lambs at both 7 and 28 days. Due to the gender dimorphism seen in adipose tissue weights the gene expression results have been shown separately for males and females. Genes were categorised into four main groups, in order to aid interpretation. These are 1)

BAT/thermogenic genes: for genes generally considered as BAT markers, or involved with thermogenesis; 2) WAT genes: for genes generally considered a marker of white adipose tissue; 3) brite genes: for genes generally associated with brite adipose tissue and 4) adipogenic/metabolic genes: genes associated with adipogenesis and/or metabolism.

5.4.5.1 BAT/thermogenic genes

Figure 5.4 and 5.5 show the effect of dietary group on BAT/thermogenic gene expression at 7 and 28 days respectively.

At 7 days there was a significant increase in *PRLR* mRNA expression in female lambs in the canola group compared with controls (p=0.0117). There was no effect seen in the sunflower group, and expression in male lambs was not affected by dietary group. There was no difference in *PRLR* expression between groups at 28 days in either gender.

Compared with controls, mean mRNA expression of *CIDEA* was increased by 12.6-fold in the canola females at 7 days (p=0.0244). Whilst there was a trend to a 9-fold increase of *CIDEA* expression in female sunflower lambs compared with controls, this did not reach statistical significance (p=0.0592). There was wide intergroup variation amongst male lambs and no clear trend with dietary group. At 28 days *CIDEA* mRNA expression showed no variation across dietary groups in either gender.

There were no clear effects of dietary group on the mRNA expression of *UCP1* at 7 days in either gender. At 28 days, the *UCP1* expression of female lambs in the sunflower group was 30% higher than controls (p=0.0125), and there were no differences seen in the female canola group. No significant differences were found between dietary groups in the male lambs at 28 days.

At both 7 and 28 days, mRNA expression of *ADRB3* was remarkably consistent across the dietary groups and genders. Finally, *DIO2* and *PRDM16* expression was also not affected by dietary group at either time point (Figure 5.5 and Figure 5.5).

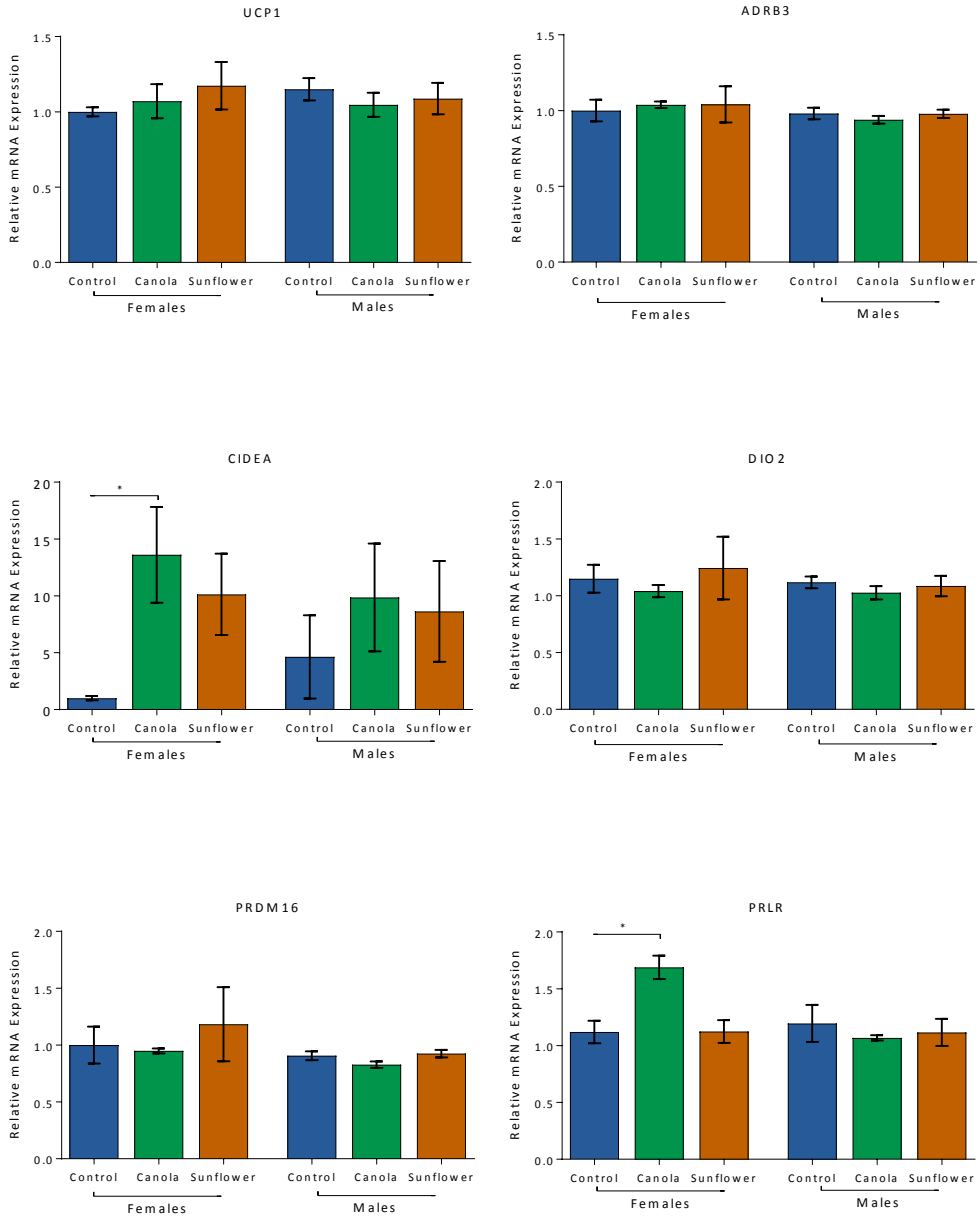

Figure 5.5 Relative mRNA expression of BAT/thermogenic genes in perirenal adipose tissue at 7 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females control n=4, sunflower and canola n=5. Males control and sunflower n=4, canola n=3 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by * p=0.0244 (*CIDEA*), p=0.0117 (*PRLR*).

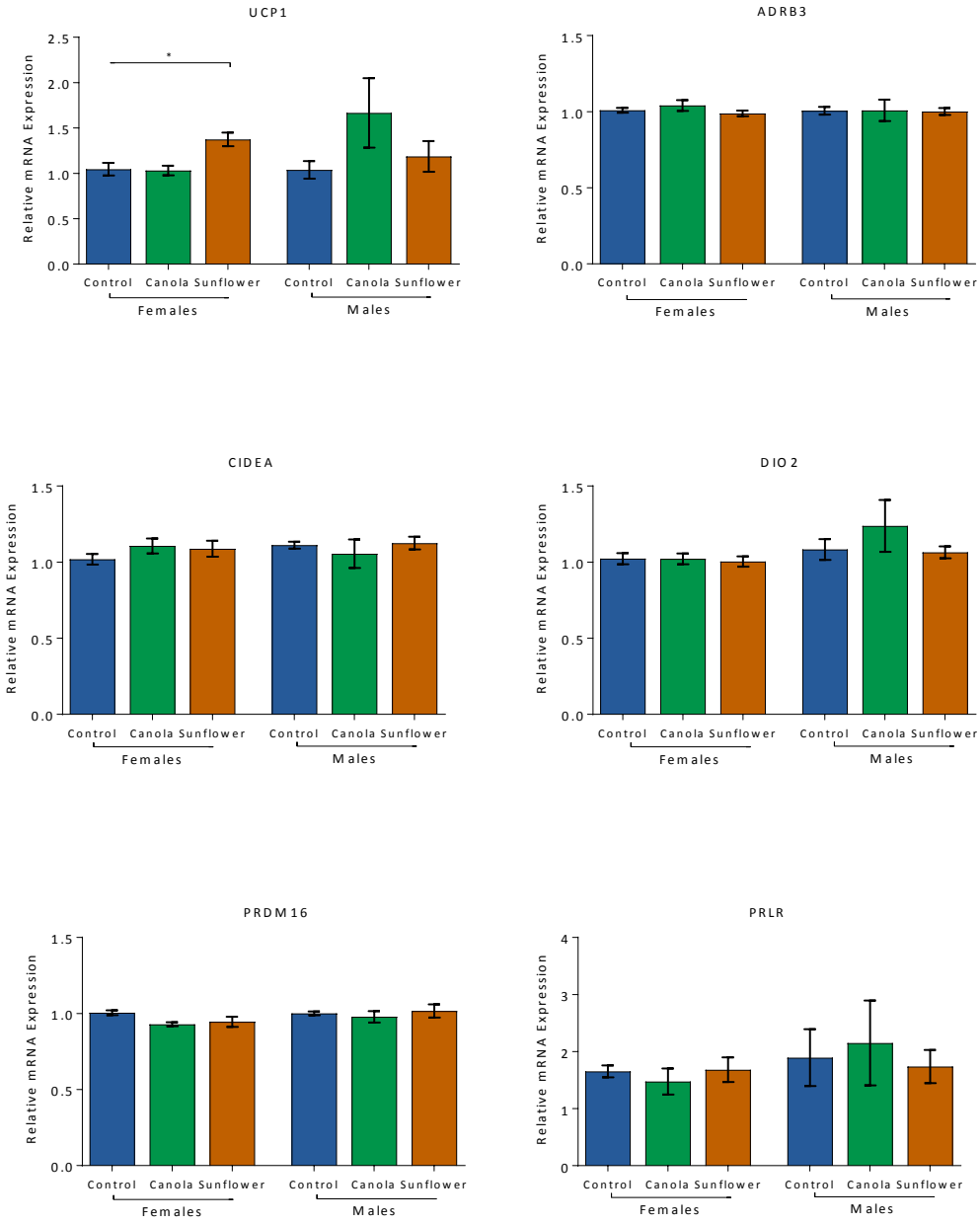

Figure 5.6 Relative mRNA expression of BAT/thermogenic genes in perirenal adipose tissue at 28 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females n=5, males n=4 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by * p=0.0125.

5.4.5.2 WAT genes

Perirenal adipose tissue mRNA expression of *leptin* and *TCF21*, two genes commonly used to identify WAT[74,134], were examined and results are shown for 7 and 28 days in Figure 5.6 and 5.7 respectively.

There was wide inter-group variation of *leptin* mRNA expression and at day 7 there appeared to be slightly higher expression in the sunflower females and lower expression in the sunflower males compared to respective controls but these were not statistically significant. At 28 days females in the canola group showed a trend for an increase in *leptin* mRNA expression compared with controls, however, this not of significance (p=0.0796). Males showed no variation of *leptin* expression with dietary group at 28 days.

At day 7, *TCF21* mRNA expression was not affected by dietary group in females. In males the sunflower group showed a trend for increase *TCF21* expression but this was not significant (p=0.0734), but there were no trends at 28 days. At 28 females in the sunflower group showed a trend for an increase in *TCF21* expression, but this was not significant (p=0.0517).

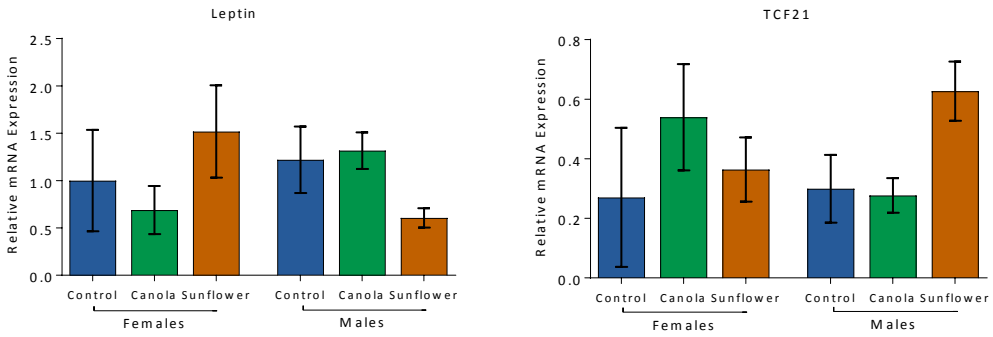

Figure 5.7 Relative mRNA expression of white adipose tissue (WAT) associated genes in perirenal adipose tissue at 7 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females control n=4, sunflower and canola n=5. Males control and sunflower n=4, canola n=3 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

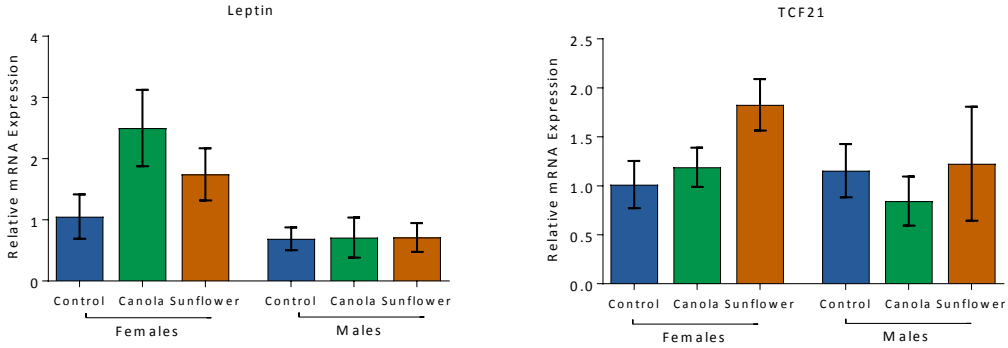

Figure 5.8 Relative mRNA expression of white adipose tissue (WAT) associated genes in perirenal adipose tissue at 28 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females n=5, males n=4 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

5.4.5.3 Brite genes

Perirenal adipose tissue mRNA expression of genes associated with brite adipose tissue at 7 and 28 days is shown in Figure 5.8 and Figure 5.9 respectively.

HOXC9 expression did not vary between dietary treatment groups at either 7 or 28 days and *SHOX2* expression results showed no significant differences between treatments at either time point.

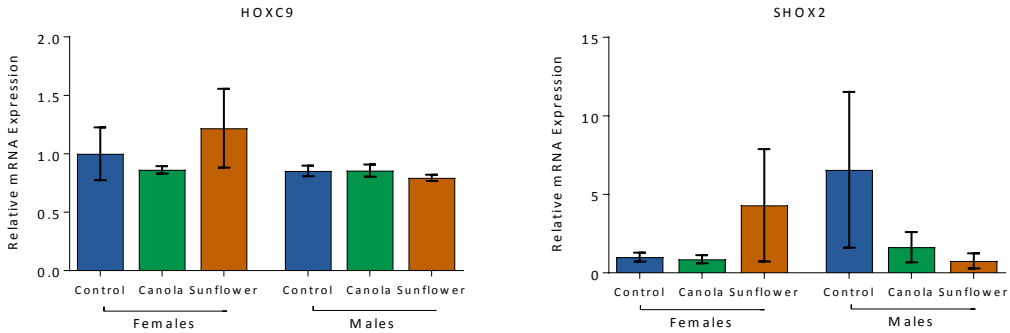

Figure 5.9 Relative mRNA expression of brite adipose tissue associated genes in perirenal adipose tissue at 7 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females control n=4, sunflower and canola n=5. Males control and sunflower n=4, canola n=3 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

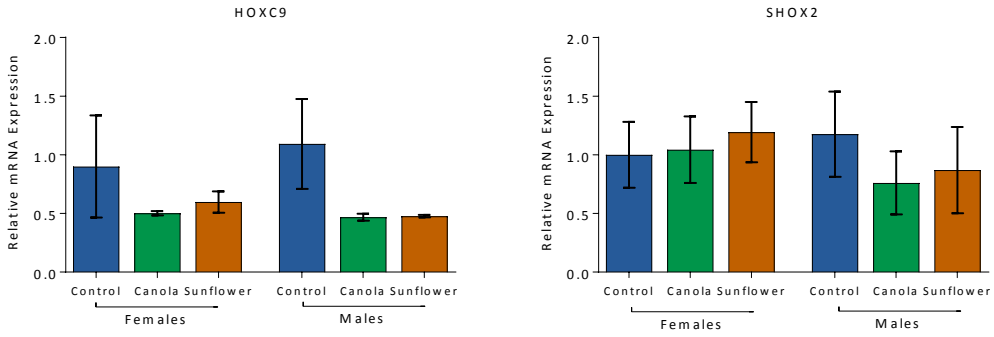

Figure 5.10 Relative mRNA expression of brite adipose tissue associated genes in perirenal adipose tissue at 28 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females n=5, males n=4 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

5.4.5.4 Adipogenic/metabolic genes

Perirenal adipose tissue mRNA expression of genes associated with adipogenesis at 7 and 28 days is shown in Figure 5.10 and Figure 5.11 respectively.

In the canola females *adiponectin* mRNA expression was 1.3-fold higher than in controls (p=0.0266) at 7 days and 0.6-fold higher at 28 days (p=0.0395). Males in the canola group showed no clear change at 7 days, but, at 28 days, their mean *adiponectin* expression showed a trend of being higher than controls, though this was not significant (p=0.0651). The sunflower lambs showed no apparent difference to controls in either males or females at either time point.

There was no significant effect of dietary group on *FABP4* expression at 7 days in either gender, but at 28 days there was a significant increase in *FABP4* expression in sunflower females compared with controls (p=0.0248). There was no effect on the canola lambs' *FABP4* expression at 28 days.

PPARγ mRNA expression showed high degrees of individual variation within groups and there were no significant differences observed across dietary groups in either gender at 7 or 28 days. *RIP140* mRNA expression was remarkably uniform between dietary groups across both genders at 7 and 28 days.

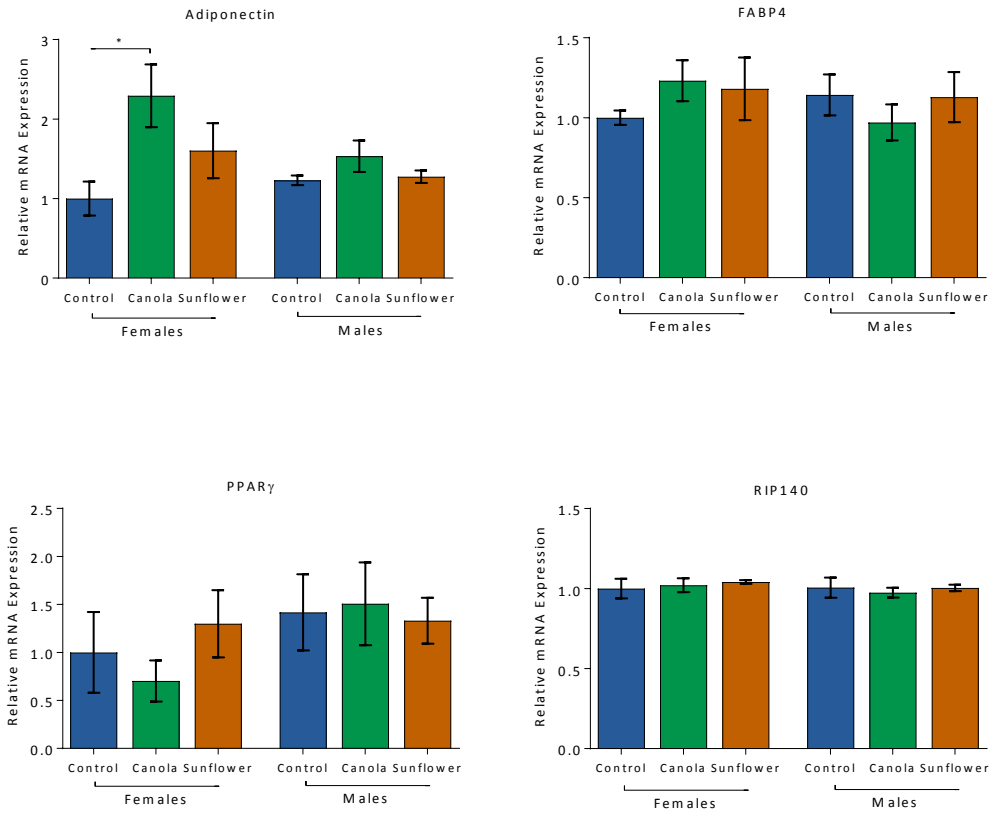

Figure 5.11 Relative mRNA expression of adipogenic/metabolic genes in perirenal adipose tissue at 7 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females control n=4, sunflower and canola n=5. Males control and sunflower n=4, canola n=3 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by *p=0.0266.

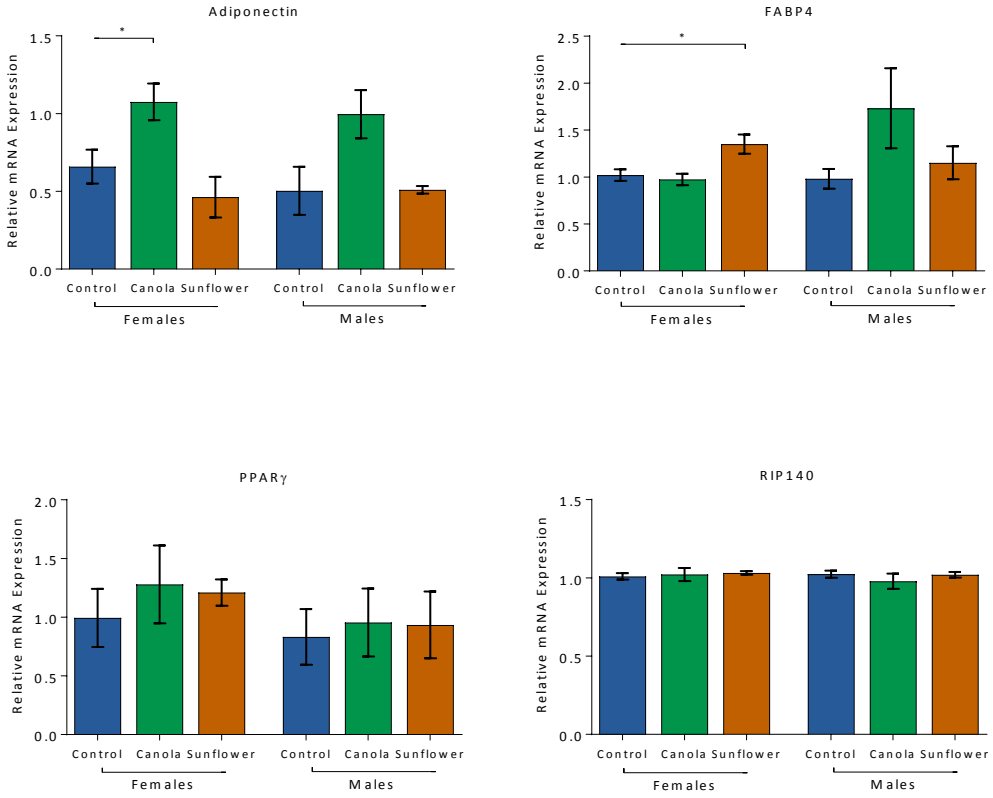

Figure 5.12 Relative mRNA expression of adipogenic/metabolic genes in perirenal adipose tissue at 28 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Females n=5, males n=4 (per group). All values are mean ± SEM. Relative expression is normalised to the reference genes *IPO8* and *RPO*. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by *p=0.0395 (*Adiponectin*), p=0.0248 (*FABP4*).

5.4.6 Histology

At 7 days, perirenal adipose tissue from lambs in all groups contained both white, unilocular and brown, multilocular cells in accordance with what we would expect in a 7 day animal[134], and there were no differences in the histological appearance of the tissue between groups (Figure 5.13).

At 28 days, the perirenal adipose tissue in control and sunflower lambs was primarily white, unilocular adipose tissue (Figure 5.16). Interestingly, the samples from the canola lambs, and especially the males, exhibited regions of multilocular adipocytes amongst the unilocular cells (Figure 5.16), which closer resembled the tissue seen in 7 day animals.

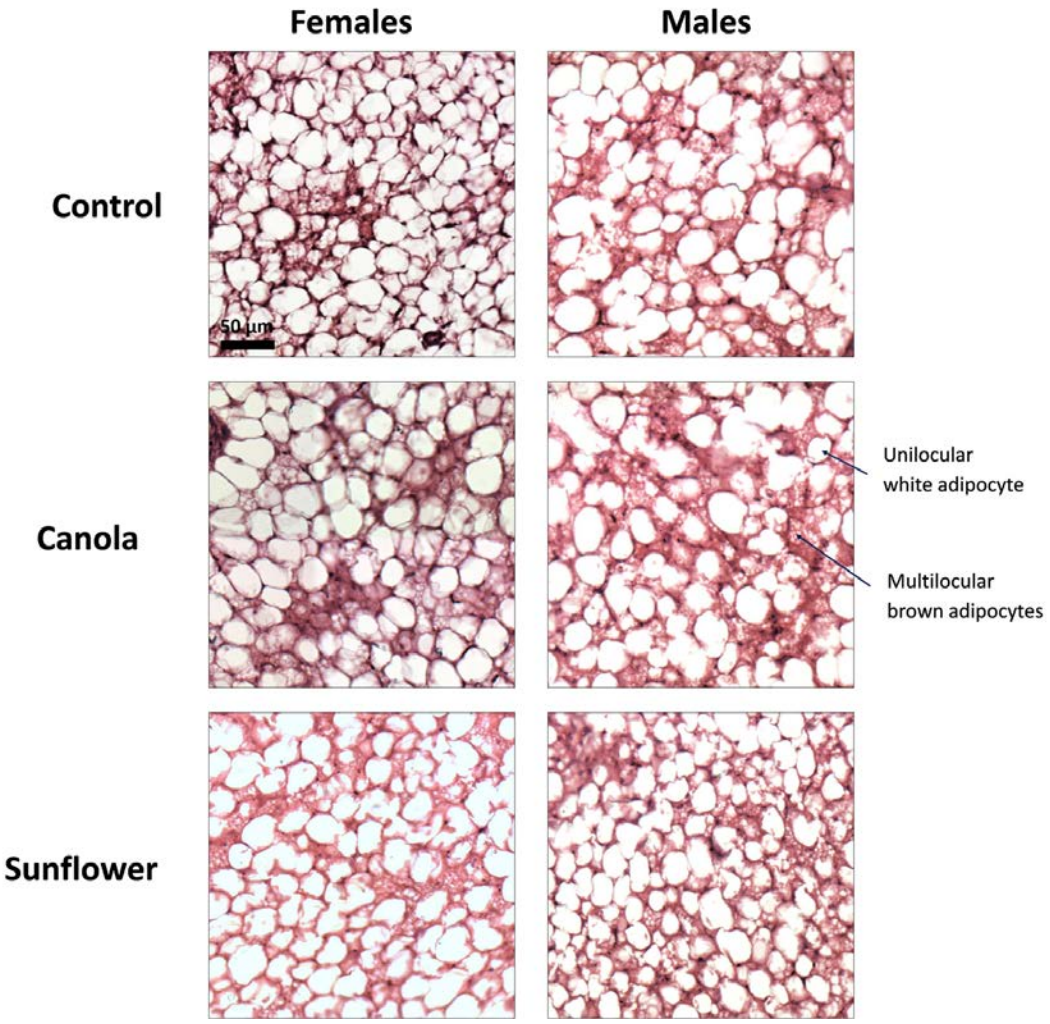

Figure 5.13 Representative haematoxylin & eosin (H&E) stained microscopic sections of perirenal adipose tissue, sampled at 7 days, at 20x magnification, on a Leica DMRB microscope.

Control: a lamb whose mother was fed a standard diet. Canola: a lamb whose mother was fed a standard diet + 3% canola oil. Sunflower: a lamb whose mother was fed a standard diet + 3% sunflower oil.

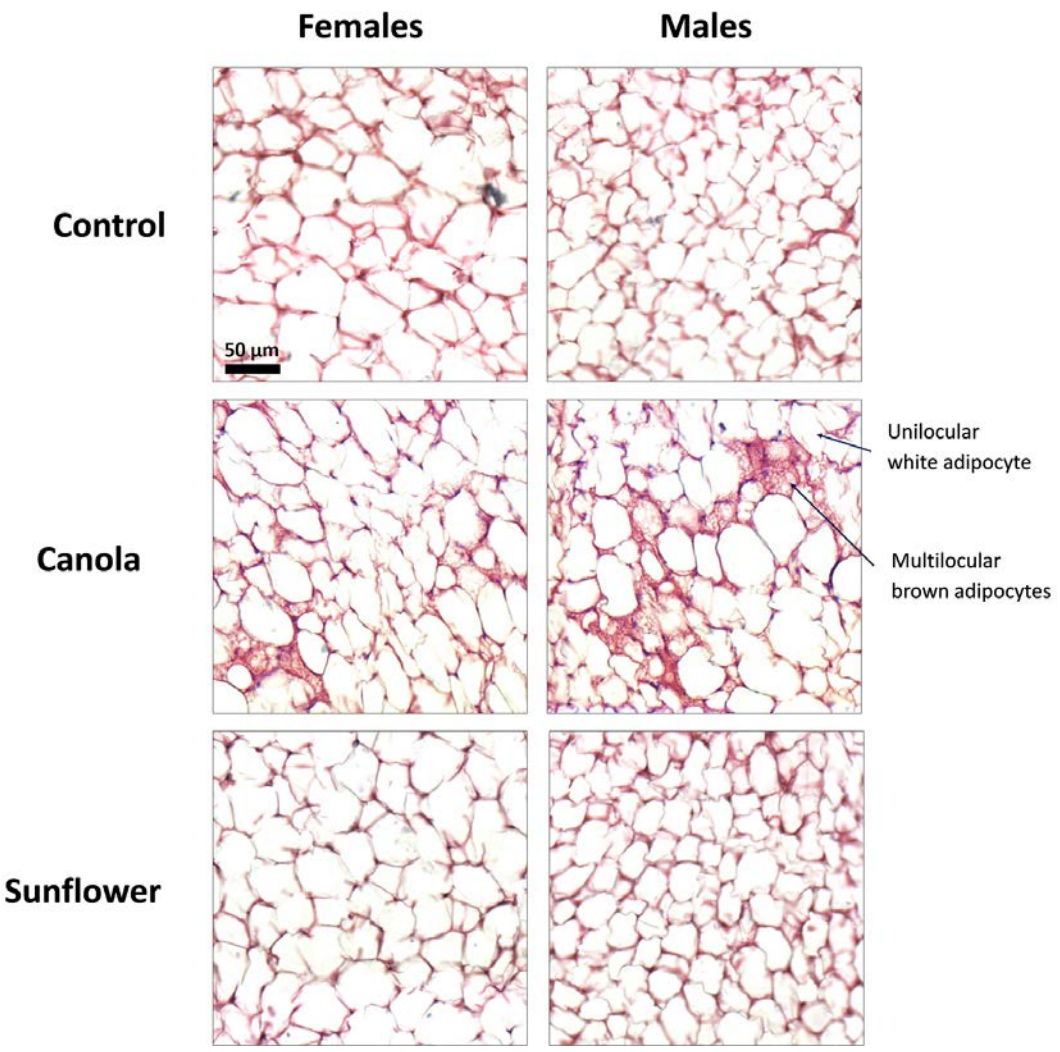

Figure 5.14 Representative haematoxylin & eosin (H&E) stained microscopic sections of perirenal adipose tissue, sampled at 28 days, at 20x magnification, on a Leica DMRB microscope.

Control: a lamb whose mother was fed a standard diet. Canola: a lamb whose mother was fed a standard diet + 3% canola oil. Sunflower: a lamb whose mother was fed a standard diet + 3% sunflower oil.

White adipocyte volume was calculated for a sample of the white adipocytes for each sample of perirenal adipose tissue (detailed in Chapter 2, Section 2.5.3, page 82). There were no significant effects seen in mean cell volume with intervention group at 7 (Figure 5.15) or 28 (Figure 5.16) days.

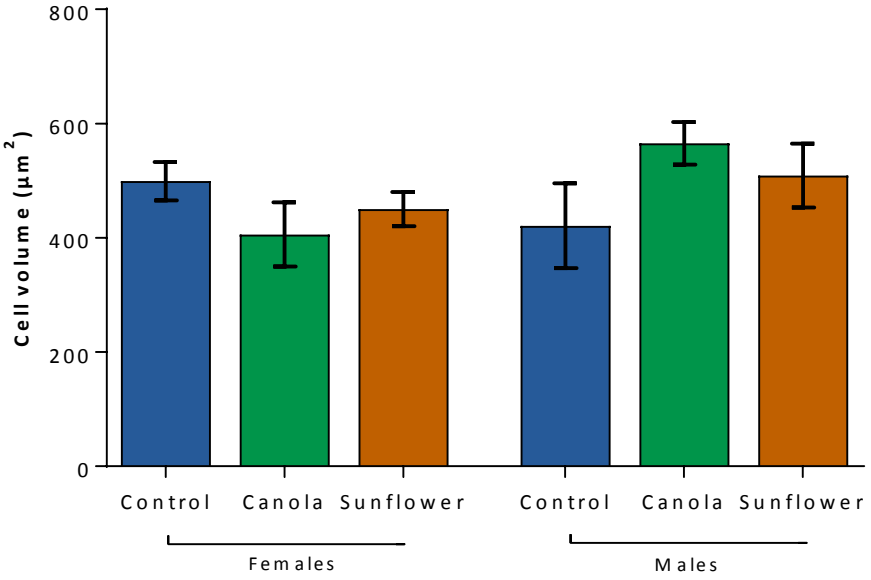

Figure 5.15 Mean white adipocyte volume (µm^2) of perirenal adipocytes at 7 days.

Dietary group relates to maternal diets, which are control (standard diet, females n=3, males n=3), canola (standard diet +3% canola oil, females n=4, males n=2) and sunflower (standard diet +3% sunflower oil, females n=5, males n=4). All values are mean ± SEM.

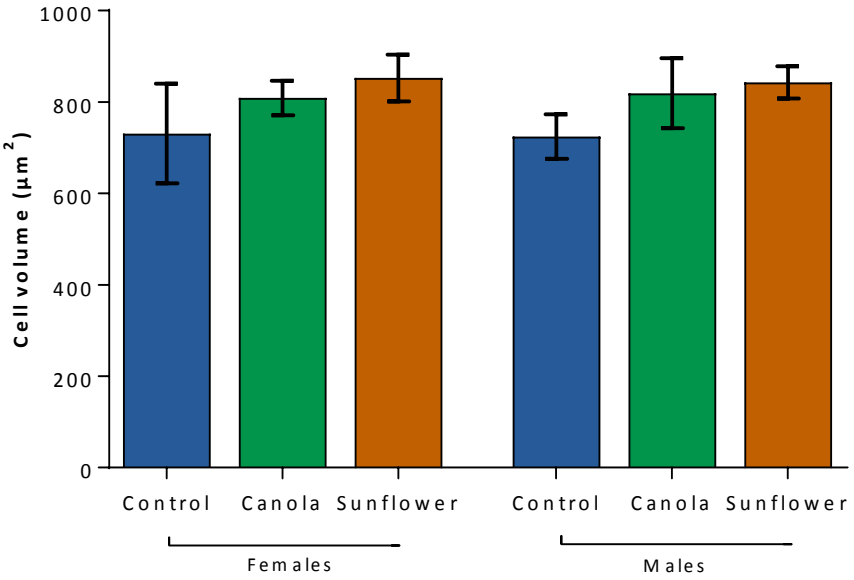

Figure 5.16 Mean white adipocyte volume (μm^2) of perirenal adipocytes at 28 days.

Dietary group relates to maternal diets, which are control (standard diet, females n=5, males n=4), canola (standard diet +3% canola oil, females n=4, males n=4) and sunflower (standard diet +3% sunflower oil, females n=5, males n=3). All values are mean ± SEM.

5.4.7 Immunohistochemistry

At 7 days there were no visible differences in the amount of staining for UCP1 in perirenal adipose tissue between dietary groups (Figure 5.17). When the percentage of staining was quantified, there were also no significant differences between controls and dietary intervention groups (Figure 5.19).

By 28 days, the perirenal adipose tissue of canola group males, and to a lesser extent, females showed areas of multilocular adipocytes stained for UCP1, which were not visible in control and sunflower group lambs (Figure 5.18).

When the percentage of staining was quantified for this time point the increases in UCP1 were significant in both female ($p=0.0439$) and male ($p=0.0238$) lambs in the canola group compared to controls. The effect was to be stronger in males, who showed a 154% increase compared to controls, whilst females showed a 79% increase. There were no significant differences between male and female controls and no effects were seen in either gender in the sunflower group (Figure 5.20).

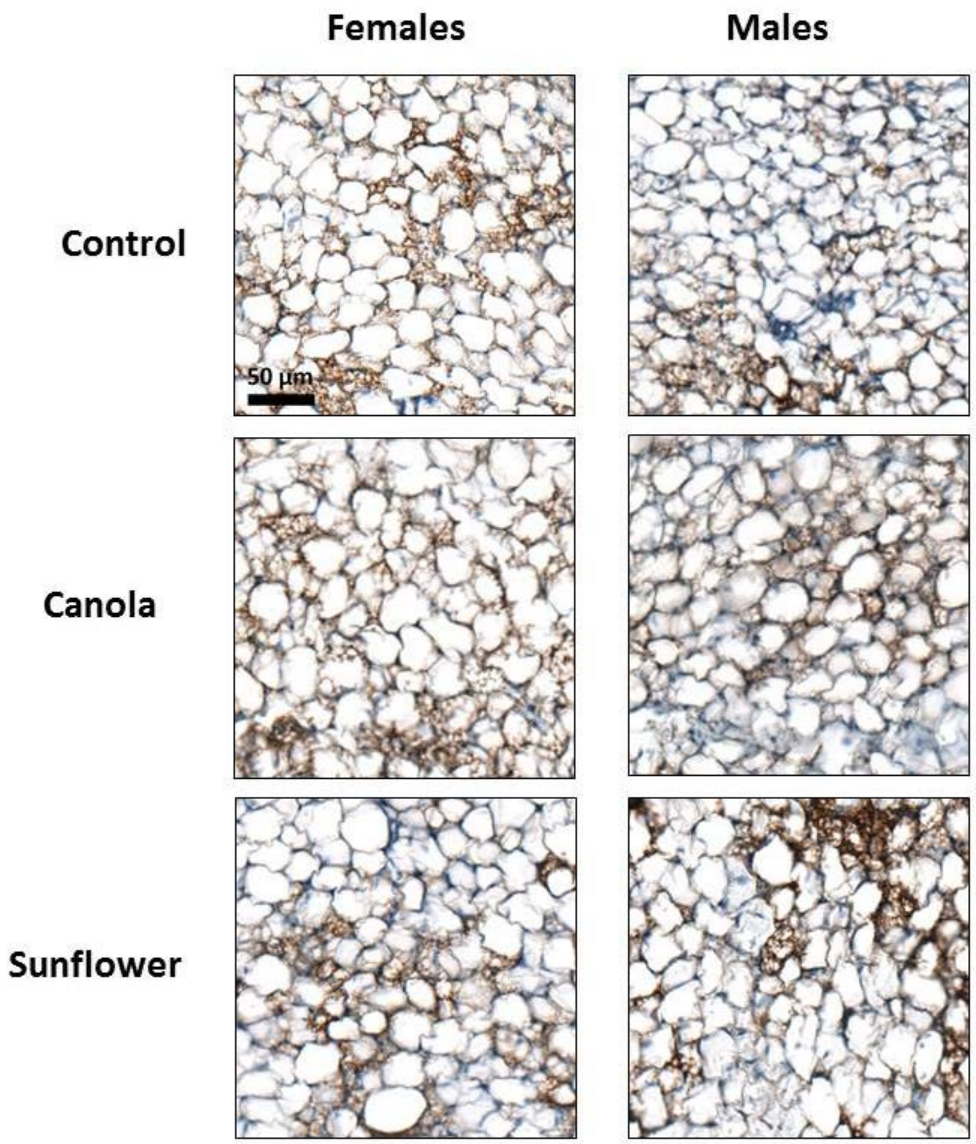

Figure 5.17 Representative uncoupling protein (UCP) 1 (1:750 primary antibody dilution) stained microscopic sections of perirenal adipose tissue, sampled at 7 days, at 20x magnification, on a Leica DMRB microscope.

Control: a lamb whose mother was fed a standard diet. Canola: a lamb whose mother was fed a standard diet + 3% canola oil. Sunflower: a lamb whose mother was fed a standard diet + 3% sunflower oil. UCP1 presence is indicated by a brown stain.

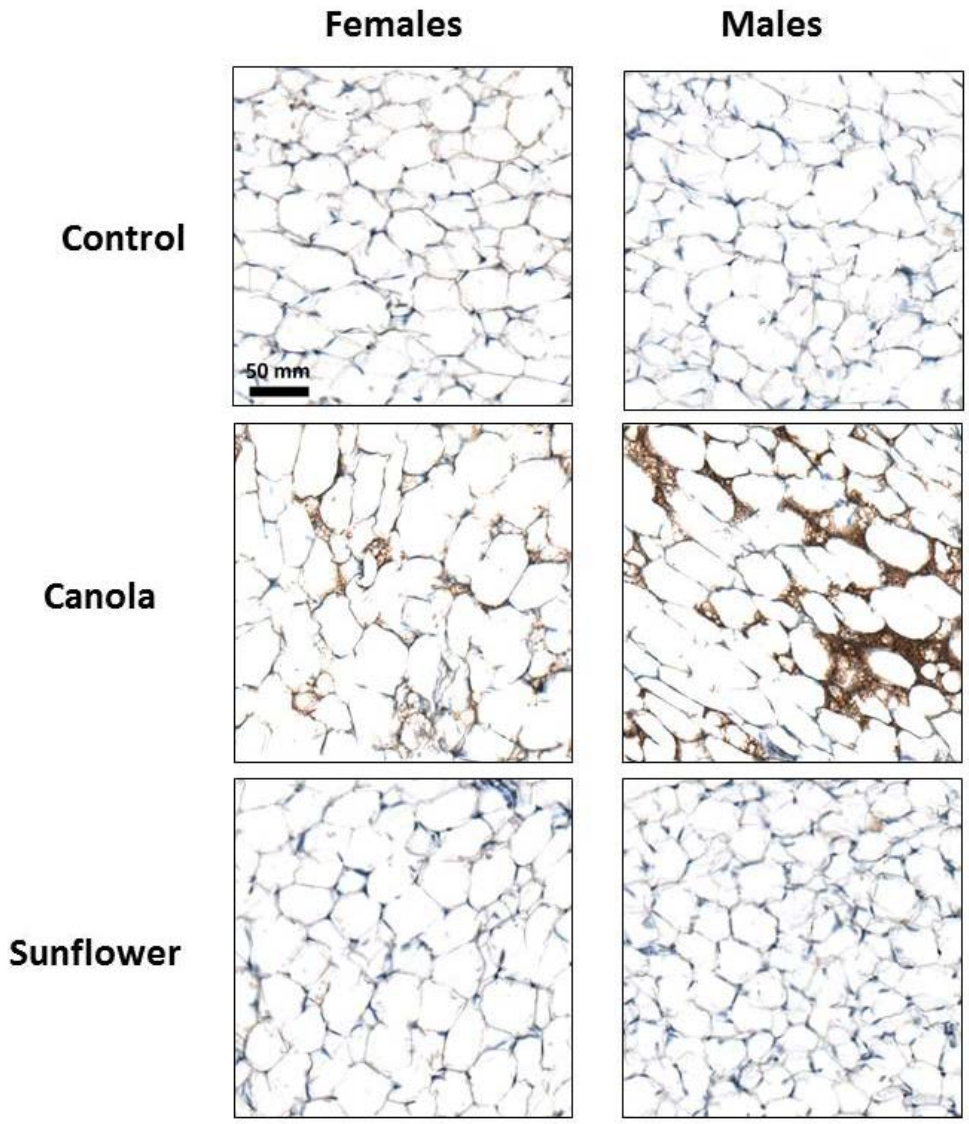

Figure 5.18 Representative uncoupling protein (UCP) 1 (1:750 primary antibody dilution) stained microscopic sections of perirenal adipose tissue, sampled at 28 days, at 20x magnification, on a Leica DMRB microscope.

Control: a lamb whose mother was fed a standard diet. Canola: a lamb whose mother was fed a standard diet + 3% canola oil. Sunflower: a lamb whose mother was fed a standard diet + 3% sunflower oil. UCP1 presence is indicated by a brown stain.

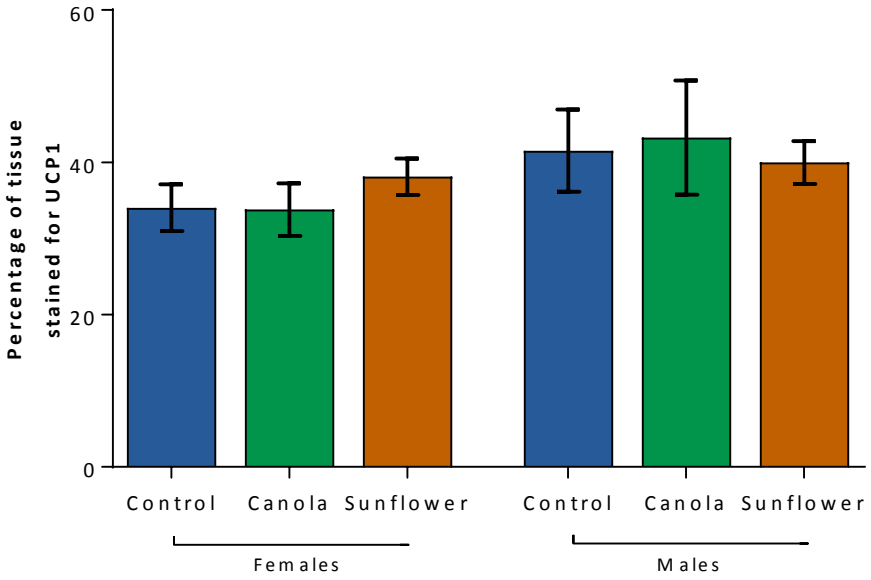

Figure 5.19 Percentage of perirenal adipose tissue stained with UCP1 antibody by immunohistochemistry at 7 days.

Dietary group relates to maternal diets, which are control (standard diet, females n=3, males n=3), canola (standard diet +3% canola oil, females n=4, males n=2) and sunflower (standard diet +3% sunflower oil, females n=5, males n=4). All values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test.

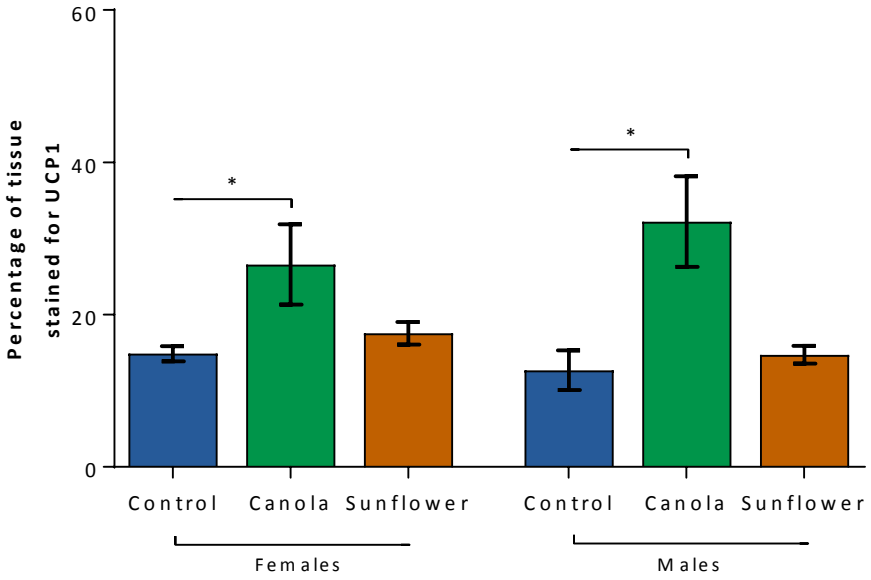

Figure 5.20 Percentage of perirenal adipose tissue stained with UCP1 antibody by immunohistochemistry at 28 days.

Dietary group relates to maternal diets, which are control (standard diet, females n=5, males n=4), canola (standard diet +3% canola oil, females n=4, males n=4) and sunflower (standard diet +3% sunflower oil, females n=5, males n=3). All values are mean ± SEM. Canola and sunflower groups were compared to the corresponding control group by unpaired t-test. Significance is denoted by *p=0.0439 (females), *p=0.0238 (males).

When viewed in terms of the decrease in UCP1 with age (Figure 5.20), which has been reported in the perirenal depot of sheep previously[100], UCP1 abundance assessed by IHC is significantly reduced in control females (p=0.0003) and males (p=0.0032), and in sunflower females($p<0.0001$) and males (p=0.0008), between 7 and 28 days. There were no significant differences between canola lambs at 7 and 28 days in either gender.

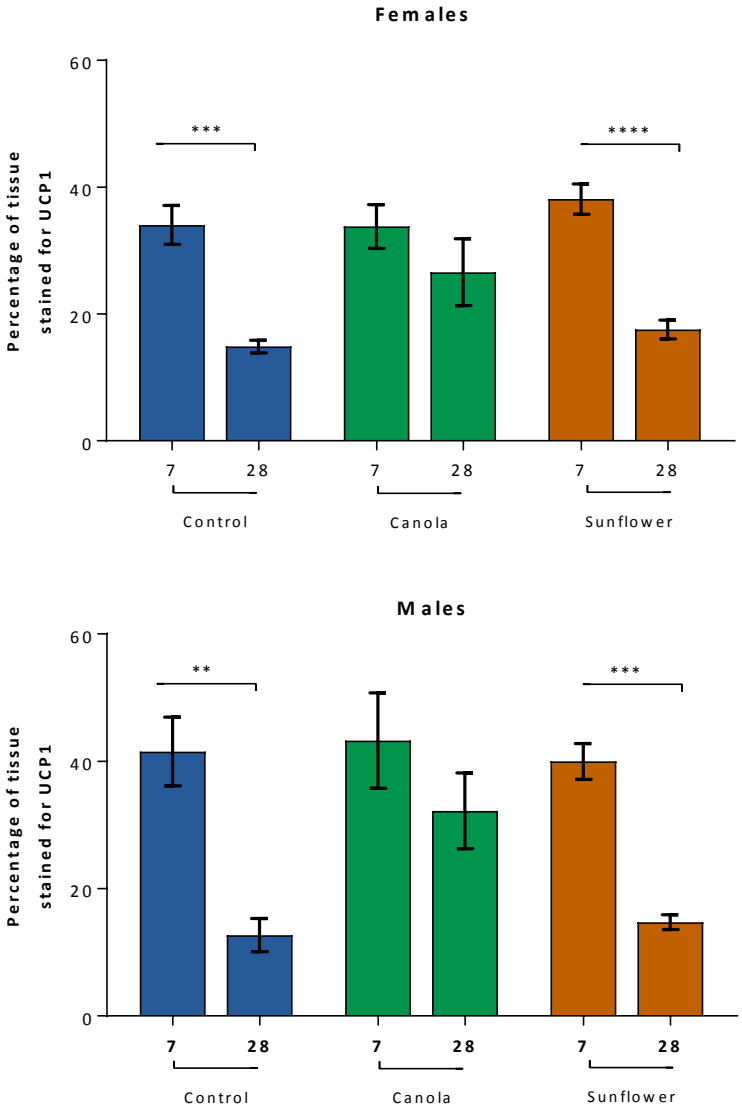

Figure 5.21 Effect of age on the percentage of perirenal adipose tissue stained with UCP1 antibody by immunohistochemistry.

Dietary group relates to maternal diets, which are control (standard diet, day 7: females n=3, males n=3. day 28: females n=5, males n=4), canola (standard diet +3% canola oil, day 7: females n=4, males n=2. Day 28: females n=4, males n=4 and sunflower (standard diet +3% sunflower oil, day 7: females n=5, males n=4. Day 28: females n=5, males n=3). All values are mean ± SEM. Day 7 vs. Day 28 groups within each dietary group by unpaired t-test. Significance is denoted by ** p=0.0032 (male controls 7 vs. 28), *** p=0.003 (female controls 7 vs. 28), ***p=0.0008 (male sunflower 7vs.28) and **** p<0.0001 (female sunflower 7 vs. 28.

5.4.8 UCP1 abundance

After finding significant differences in the distribution of UCP1 in 28 day perirenal adipose tissue samples from the canola group lambs, when compared with controls, I performed Western blot analysis on these samples in order to get another quantifiable measure of the UCP1 protein abundance. The day 7 samples and sunflower group samples were not included due to there being no distinguishable differences between the histological appearance of the tissue, or the presence of UCP1 (by immunohistochemical analysis) compared with controls. This also allowed all samples to fit on one gel, eliminating the potential confounders which can occur when making comparisons between samples run on separate gels. The Western blot gel is shown (Figure 5.22), where UCP1 can be seen at approximately 32kDa.

Figure 5.22 Representative image of Western blot with primary antibody for uncoupling protein 1 (UCP1, 32kDa).

I found that relative UCP1 abundance in the canola group males was a significantly higher than controls (p=0.0026, Figure 5.21). However, mean UCP1 in the canola group females was not significantly higher than controls (p=0.164, Figure 5.21). Whilst IHC results showed a significant increase in the canola females UCP1 abundance at 28 days, which was not confirmed on Western blotting, the results from both assays show similar trends. It is likely that the lack of statistical significance in the female lambs is due, at least in part, to the low sample sizes.

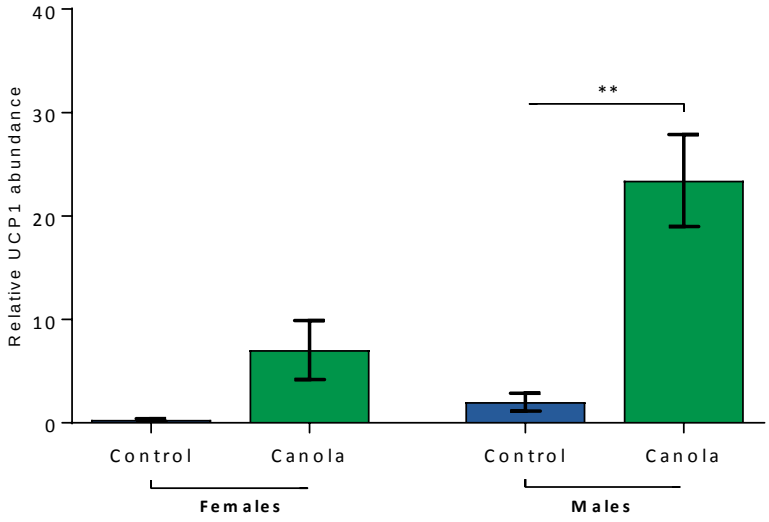

Figure 5.23 Relative UCP1 abundance in perirenal adipose tissue at 28 days by gender and dietary group.

Dietary group relates to maternal diets, which are control (standard diet) and canola (standard diet +3% canola oil). Females, control n=2, canola n=3. Males, control n=4, canola n=3. All values are mean ± SEM. Comparisons were made between control and canola for each gender, by unpaired t-test. Significance is denoted by, ** p=0.0026.

5.5 Discussion

5.5.1 Maternal canola oil supplementation increases UCP1 abundance in male and, to a lesser extent, female offspring

Previous work has shown that nutritional restriction during pregnancy can affect the *UCP1* gene expression of offspring[546,547], these studies have focussed on caloric intake rather than specific nutrients. I aimed to investigate whether supplementation during lactation and consequent alterations in offspring's early life diet had the potential to alter their BAT development. There have also been studies showing that individual fatty acids may play a role in the activation of BAT. These include CLA[331,332], EPA and DHA[193]. These fatty acids were all measured in the milk in my study yet no significant effects were seen in milk EPA and DHA content, and CLA will be discussed further on (Section 5.5.1.1). My results indicate that maternal supplementation of canola oil prevents or delays the loss of the offspring's UCP1, especially in males.

The cause of this apparent gender effect is not clear. Small animal studies tend to suggest that females have more BAT than males[548,549] and that males lose their BAT more rapidly than females[550]. Yet Rodriguez *et al*. found that whilst females have a greater thermogenic capacity[548] when fed a high fat diet, males exhibit a greater increase in UCP1[548]. Their study found that females showed a lesser increase in *ADRB3* expression as a result of a high fat diet compared to males who, therefore, showed a greater capacity to respond to the high fat diet. Whilst no effect of dietary group on *ADRB3* mRNA expression was demonstrated in the study reported here, its protein abundance was not assessed and, therefore, it is possible that similar mechanisms could be responsible for the male lambs exhibiting a greater response than females to the alterations in the milk FA profile induced by maternal canola oil supplementation.

Whilst my IHC results showed significantly more UCP1 present in both male and female lambs in the canola group at 28 days, my results from Western blotting only showed significance in the male animals at this time point. The mean of the female canola group lambs was numerically, but not significantly higher than control females. The lack of statistical significance is likely to be due to the low sample size number of female lambs (controls, n=2, canola, n=3). A retrospective power calculation was performed, and it stated that 7 lambs would be required per group (control and canola) in order to yield a significant result ($p<0.05$) with a statistical power of ≥80%.

5.5.1.1 The potential role of CLA in the increase of UCP1

I hypothesised that the changes in milk fatty acid profile induced by maternal dietary supplementation of canola oil and sunflower oil, in particular the increased CLA concentrations, would result in an increase in UCP1 abundance in offspring. CLA has been reported to affect UCP1 (Chapter 1, Table 1.2, Page 36) but due to some conflicting studies, firm conclusions and mechanisms are yet to be reached. In my study, the milk of both canola and sunflower supplemented ewes contained higher levels of total and *cis*-9, *trans*-11 CLA than that of controls at 28 days. In addition to this, the milk of sunflower supplemented ewes showed a large increase in the *trans*-10, *cis*-12 isomer compared with controls. This isomer was unaffected by canola oil supplementation.

A range of other CLA isomers were also tested, and generally increased with supplementation, but little biological significance has been attributed to these isomers. Therefore, my investigation is largely focussed on the two best characterised, biologically active isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12.

It is likely that numerous factors in the milk lipids – and possibly other nutrients worked together to cause the changes observed in offspring, however, it is possible that CLA was involved. As my study showed increases in *cis*-9, *trans*-11 in both supplemented groups' milk, but increases in *trans*-10, *cis*-12 in only the sunflower group milk, it is possible that when the *cis*-9, *trans*-11 isomer is increased without an accompanying increase in the *trans*-10, *cis*-12 isomer, then it may promote *UCP1*. When the increase in maternal milk *cis*-9, *trans*-11 is accompanied by increased *trans*-10, *cis*-12, however, this effect may no longer be present and may potentially result in less UCP1, in their lambs. There is some evidence in the literature to support this. Both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA have been shown to increase *UCP1* mRNA and protein when administered separately[97,330,331], yet one study found that whilst *cis*-9, *trans*-11 CLA alone increased *UCP1* mRNA, the *trans*-10, *cis*-12 isomer reduced it[332]. When the two isomers are fed together as a mixed supplement (approximately 50:50 ratio), results have been inconclusive, with some studies showing an increase in UCP1 in rats[337] but not mice[316,335,337], and others reporting no effect[317,319]. This alludes to the possibility of a complex relationship between the two isomers and their potential to alter *UCP1*. House *et al.*[330] found a 12-fold increase in *UCP1* mRNA in mice fed a 1% supplement of *trans*-10, *cis*-12 CLA, however, protein abundance was not analysed. A similar study also found that a dietary *trans*-10, *cis*-12 supplement increased *UCP1* mRNA in mice[97]. Again, protein analysis was not carried out.

It is worth noting that there was a significant increase in *UCP1* mRNA in the sunflower group females (who received milk with increased *trans*-10, *cis*-12 CLA) at 28 days, but did not see a corresponding increase in UCP1 itself. It is fairly well understood that mRNA expression does not usually directly correlate with protein abundance[551], and many factors can affect the process between transcription and translation which may mean that mRNA and protein differ[339]. It is possible that the *trans*-10, *cis*-12 isomer could increase transcription (mRNA) but not translation (protein). When considered in this context, the majority of (the few) studies looking at isomer specific effects on *UCP1*, have used mRNA as their outcome measure.

5.5.1.2 The significant increase in milk erucic acid content could be linked to corresponding UCP1 increases in the canola group

As previously stated, it is not possible to attribute the retention of UCP1 observed in my investigation to any one fatty acid present in the milk. However, the omega-9 MUFA erucic acid and UCP1 were both increased in the canola group at 28 days. However, in Chapter 3,

Study A, the milk erucic acid content was also raised with canola oil supplementation, yet there were no changes in female offspring's perirenal adipose tissue UCP1 abundance, suggesting that the elevated milk erucic acid was not the cause of the delay in the loss of UCP1. In addition, to my knowledge, there is no published evidence of the effect of erucic acid on BAT activity. Whilst this may potentially be of interest for further research, even if erucic acid is involved in delaying the loss of UCP1, it may not be beneficial to pursue as a potential therapeutic intervention as erucic acid has been associated with negative health effects. For example, dietary erucic acid supplementation in rats has been associated with chronic doxorubicin toxicity[552], increased myocardial necrosis[553] and cardiac lesions[554]. Due to these safety concerns, efforts have been made to reduce the erucic acid content of rapeseed oil for human consumption, which resulted in canola oil superseding traditional rapeseed varieties in the 1980s[519]. In light of findings, a tolerable level of human exposure has been established (with a 120-fold safety margin) of 500 mg erucic acid/day for the average adult[519]. Erucic acid could be even more damaging in early life, as nursing piglets had a lower tolerance before presenting myocardial lipidosis[555], and for this reason infant formula milk erucic acid must not exceed 1%[515,556]. For these reasons, even if there is a relationship between erucic acid and *UCP1*, there is unlikely to be a translational benefit.

5.5.2 Effect of altered milk fatty acid profile on the expression of genes in the perirenal adipose tissue

5.5.2.1 BAT/thermogenic genes

At 7 days of age, there was little variation between the dietary groups in the perirenal adipose tissue mRNA expression of genetic markers of BAT and thermogenesis. This is likely to be attributable to the fact that the effects of supplementation on the milk FA profile were not as pronounced at 7 days. Amongst the BAT/thermogenic genes, the only significant results at this time point were in *PRLR* and *CIDEA* mRNA expression in canola female lambs. PRLR plays a critical role in promoting thermogenesis[557] and regulating UCP1 function[94]. Pope *et al.* studied *PRLR* and *UCP1* in sheep and demonstrated that *PRLR* mRNA abundance peaked shortly before a peak in *UCP1*[100], suggesting that increased *PRLR* precedes an increase in *UCP1*. The loss of PRLR in young sheep has been shown to be highly correlated with the loss of UCP1[94], and prolactin administration has been shown to increase UCP1[95]. This suggests that the elevation in *PRLR* expression at 7 days in the canola group females may have played a role in the higher UCP1, compared with controls, at 28 days. However, the lack of significant changes in *PRLR* at either time point in the canola males,

despite them displaying an increase in UCP1 protein at 28 days (to a greater extent than females) suggests that *PRLR* is not the main driver of this effect.

CIDEA is an established marker of BAT in rodents[558] and *CIDEA* null mice are lean and resistant to diet-induced obesity[96]. Whilst its expression seems to mirror that of *UCP1*, paradoxically, it appears to inhibit thermogenesis[96]. Zhou *et al*. found that *CIDEA* null mice exhibited a greater thermogenic activity and lipolysis in BAT and their findings, along with their further experimentation in yeast, provided strong evidence that CIDEA binds to UCP1 and inhibits its activity[96]. *CIDEA* appears to be species specific, and is expressed in WAT not BAT in humans[98]. It has not been fully characterised in sheep.

The significant increase of *UCP1* mRNA in sunflower females at 28 days is not mirrored in any of the other thermogenic genes tested and a clear genetic pathway to explain this increase is not readily apparent. The increase in *UCP1* expression was small and was not translated to an increase in UCP1 when assessed by immunohistochemistry. This leads me to suggest that this result may be of little functional significance.

5.5.2.2 Variable results between *UCP1* mRNA and protein

Interestingly, the *UCP1* mRNA results did not correspond to those I have found in protein abundance assessed by IHC or Western blotting in the canola group lambs. Whilst it is often the assumption that an increase of mRNA expression of a gene is indicative of an increase in the functional protein, when investigated further, this is not as clear cut as it seems. In fact, the use of mRNA profiling has been criticised for the transcriptome not dependably representing the proteome[338] and Anderson *et al.* reported that the correlation coefficient between mRNA and protein abundances in liver samples was 0.48[559] and proposed a number of potential reasons for these relatively weak correlations. Including, cellular control systems which cannot be observed in mRNA, due to operating entirely in the protein domain and the fact that protein is more stable than mRNA in most tissues[560], and *UCP1* mRNA has been reported to degrade at a faster rate than the protein[561], although great care was taken in my study for tissue to be snap frozen immediately after collection, and tissue preparation for RNA extraction was carried out in a time-cautious manner. Finally, protein abundance is a much better measure of function than mRNA. By its very nature, mRNA is simply a messenger whereas proteins perform numerous biological functions. Therefore protein measurement is much more direct and whilst I have carried out and presented mRNA analysis on *UCP1* in my study, I consider this to be superseded by my work on the UCP1 itself Unfortunately, there are a lack of commercially available

antibodies for use in sheep tissue, so all of my other gene work has been carried out using mRNA expression.

This is not to say that mRNA work is not, in itself, useful, as other studies[562] have found better correlations than Anderson *et al.*[559]. However, as I had measures of both mRNA and protein, in this case protein is the more reliable measure.

5.5.2.3 WAT genes

I used mRNA expression of *leptin* and *TCF21* as markers of WAT. The *leptin* gene transcribes the adipokine leptin which is synthesised within and secreted from white adipocytes[563]. *TCF21* is highly expressed in WAT compared to minimal expression in BAT or brite adipose tissue[74].

The results for *leptin* mRNA expression were not significant, despite changes in the plasma leptin amongst canola and sunflower group females. Although, this is not entirely unexpected, as it is known that short-term secretion of leptin does not always cause alterations in *leptin* mRNA expression[564].

5.5.2.4 Brite genes

HOXC9 has been used as a marker of brite adipose tissue, although, it is also expressed to a lesser extent in WAT[74]. Therefore its use as a marker of brite adipose tissue, especially in mice, has been contested[565]. Nevertheless, as it is not expressed in classical BAT[74], it can be useful as a marker to distinguish classical BAT from brite adipose tissue. In my investigation, I used it for this purpose, to indicate whether the higher abundance of UCP1 and multilocular adipocytes resulted from retained classical BAT or from recruited brite adipose tissue. I also used *SHOX2* as a marker of brite adipose tissue, as Walden *et al.* found it to be highly expressed in brite adipocytes of mice with minimal expression in BAT and WAT[74].

The lack of change in *HOXC9* and *SHOX2* with dietary intervention indicates that the elevated levels of UCP1 in the canola group lambs are not as a result of 'browning' but more likely a delay in, or prevention of, the loss of classical BAT. This poses the obvious question of whether the increased UCP1 abundance in the lambs in the canola group would be retained later in life. To address this, further studies would be required.

5.5.2.5 Adipogenic/metabolic genes

Adiponectin has been shown to inhibit expression of *UCP1* in BAT in mice by suppressing *ADRB3* expression[102]. Whilst *adiponectin* mRNA expression was increased in the canola females at both time points and showed a trend for being higher in males at 28 days, there was no apparent suppression of *ADRB3* or *UCP1* in these lambs compared with controls. Even if the increase in adiponectin had caused some suppression of *UCP1* mRNA, this was not reflected in the protein as UCP1 abundance was significantly higher in canola group lambs. This could offer an explanation as to why canola females showed a lesser increase in UCP1 than their male counterparts. It could be that the common factor in the milk which led to the UCP1 increase was partially offset by the accompanying increase in *adiponectin* mRNA expression in the female lambs. The reason for the gender specific effect of *adiponectin* in the canola group is unclear. In human pubescent adolescents[566] and adults[567], but not in prepubescent children[104,566] plasma adiponectin is higher in females compared to males. This would not explain why gender differences were not seen amongst controls.

CIDEA expression is controlled by *PPARγ* and has been shown to be upregulated by *PPARγ* agonist treatment in mice[568] and is known to be suppressed by *RIP140*[569]. However, although there was an increase in *CIDEA* expression in canola females at 7 days, I saw no accompanying effects on *PPARγ* or *RIP140*.

FABP4 is often used as a marker of differentiation in adipocytes[144]. The expression of *FABP4* was increased in the female sunflower group lambs at 28 days, in line with the increase in adipose tissue weight and growth rates seen in this group. *FABP4* null preadipocytes exhibit both hyperplasia and hypertrophy along with increased expression of *PPARγ*[570], and *FABP4* null mice show increased adiposity[571]. The female lambs in my study, whose mothers received a sunflower oil supplement during lactation, did not appear to exhibit hypertrophy based on histological analysis, therefore, their increased adipose tissue depot weights suggest hyperplasia. This could potentially be the driver of increased expression of *FABP4,* which has also been associated with insulin resistance[572,573], thought to be related to *FABP4*'s effect on *PPARγ*. PPARγ is associated with increased insulin sensitivity/protection from insulin resistance[574-576]. Activated PPARγ is a transcription factor of *FABP4*, however, when expressed *FABP4* negatively regulates PPARγ levels in adipocytes[570].

5.5.3 Increased adiposity and growth rates in lambs may be related to elevated milk omega-6 fatty acids

There are several potential reasons for increases in adiposity and growth rates of the female lambs in the sunflower group and the smaller, (largely insignificant) increases in individual fat depot weights of canola group females (accumulating to a significant increase in total adipose tissue). The first is calorie intake. Whilst my investigation did not measure total milk energy content, it showed no change in milk fat content with dietary group. As fat is the macronutrient highest in energy, it is likely that there was not a significant increase in milk energy content. However, it is possible that lambs in one group were consuming more milk. My study did not measure lamb's milk intake, therefore I cannot be certain that intakes did not vary between groups by chance. There is research to suggest that mothers may have a gender bias, investing more in one gender, although this seems to favour males who, Duncan *et al*'s work in horses suggests, tend to weigh more and receive more milk[577]. Research specific to sheep around gender variation in milk intake is sparse, but suggests that there does not seem to be more investment in one gender[578]. The gender bias theory falls short at addressing the lack of differences in weight gain in relation to gender in control lambs, so I propose that it is more likely that the effect on adiposity is down to changes in milk composition which are elicited by maternal supplementation, which may have gender specific effects. Another theory I considered was the possibility that mothers raising males would produce milk of a different composition to those raising females, as there is some evidence of this in the literature[579], however, when I investigated this, it did not appear to be the case and was unlikely to influence my study due to many of the ewes in my study raising M/F twins.

Omega-6 PUFA concentrations were generally higher in the milk of sunflower supplemented ewes compared with controls. In particular, the omega-6 LA increased in the sunflower group milk. This effect was not seen in the canola group, in fact milk from canola oil supplemented ewes was generally lower in omega-6 fatty acids compared with controls. Milk content of AA (a derivative of LA) was lower at 7 days in both the canola and sunflower oil group's milk, compared with controls. By 28 days, there was only a decrease in the milk AA content of the canola supplemented ewes. This could help explain the smaller increase in fat mass seen in the canola females. Piglets supplemented with AA showed a 27% increase in body weight without changes in length, in just 2 weeks of supplementation[288], this increase being partially due to an increase in bone mineral density (BMD). In mice, mothers fed a high fat diet containing high LA (also present in high quantities in sunflower

oil, and to a lesser extent canola oil, during gestation and lactation produce offspring with increased fat mass[286] and young mice fed a diet high in LA showed an increase in body fat[289]. *In vitro* LA, and its derivative AA, have been shown to promote differentiation of preadipocytes, increase lipogenic gene expression, increase fat mass, and increase adipocyte number and size[282,289,522,523]. This offers a likely explanation for the increase in adiposity observed in sunflower group female lambs in my investigation, but does not explain why males (receiving the same milk) did not show this effect, which is discussed further on in this section.

UCP1 abundance was higher in the perirenal adipose tissue of canola group lambs. This would result in higher energy expenditure if *UCP1* were activated[580]. It is a possibility that this prevented the canola group females exhibiting similar weight gain to that seen in the sunflower females. However, the males in the canola group showed a larger increase in UCP1 than the females, yet I did not see lower relative adipose tissue weights in the canola group males compared to controls.

Omega-6 fatty acids may promote adipogenesis, potentially through an increase in endocannabinoid activity[581]. Two of the best characterised endocannabinoids are 2-AG and AEA, which are both derived from omega-6 AA and elevated by dietary LA[581]. Increased endocannabinoid levels induce hyperphagia due to impaired *leptin* signalling[582], so this may fit in with the increases in plasma leptin observed in sunflower group females, however, this would not explain why increases were seen in the plasma leptin concentrations of the canola group females too. In addition to affecting appetite, endocannabinoids can also lead to obesity as they have been shown to increase *de novo* lipogenesis and peripheral adiposity[581,583], which offers an explanation for the increase in adiposity seen in treatment group females.

This does not explain why only the female lambs exhibited increased adiposity. Human females[584,585] and lambs[460] possess more adipose tissue than males. Whilst adiposity was increased in female canola group lambs' in the absence of elevated dietary LA, suggesting that other factors are playing a role, my theory of increased dietary omega-6 causing elevated endocannabinoids and thus increased circulating leptin and adiposity in sunflower females is plausible, although it does not explain why it is not occurring in their male counterparts. This leads me to postulate that male lambs may have a less efficient conversion of AA to endocannabinoids. Indeed, there do appear to be gender specific effects in endocannabinoids[586]. CB_1 receptors are one of the two identified cannabinoid

receptors and are located in the brain[587]. Female rats show an earlier peak in levels of CB_1 receptors (30 days of age) compared to males (40 days of age)[586], so it is possible that female lambs at 28 days of age have higher levels of CB_1 receptors than males and that this, paired with an increase in endocannabinoids induced by the increased levels of dietary omega-6 fatty acids present in the sunflower group milk, results in females in the sunflower group having increased appetite (leading to increased dietary intake and adiposity) and lipogenesis.

As well as animal investigations, there is evidence in humans to suggest that omega-6 fatty acids play a role in obesity. In the USA, the percentage of infants aged 6-11 months who are >95th centile nearly doubled between 1976 and 1994[588]. In such young infants, it is not plausible to suggest that this is simply down to an increase in fat and energy intake alongside inactivity. One theory is that the increase seen in maternal LA intake, and subsequent increase in breast milk LA content[290], could play a role in increasing adiposity of their offspring. An association between tissue AA and BMI has been seen in children[589]. Finally, an interesting observation has been made by Ailhaud *et al*.[290], who observed that childhood obesity rates are higher in the USA than in Europe, yet when comparing the energy intakes of infants (1-2 years old) from the USA and France in the 1980s, few differences were seen in macronutrient intakes, but the percentage of PUFAs (likely to be omega-6) were 1.5-fold higher in infants from the USA.

The increased adiposity seen in sunflower group females in early life has the potential to be damaging to their long-term health, especially if it was maintained. *In vitro* research shows that exposure to increased omega-6 fatty acids in early life, a critical time for development, could potentially result in a permanent increase in the number of adipocytes, thus increasing their propensity to store adipose tissue[282,522]. The lambs in my study were in a critical window for growth. In humans[590,591] and lambs[592], the most rapid growth occurs in the first few weeks after birth. Therefore, factors which accelerate this growth, such as the maternal sunflower oil supplementation in my study, could permanently affect long-term health[282,522]. A study in rats found that overfeeding in the postnatal period led to accelerated weight gain and then higher body weight gain until adulthood along with an increased risk of CVD and diabetes[593]. Other rodent studies have shown that early postnatal overfeeding increases risk of obesity, CVD, metabolic disease and renal diseases in adulthood[594-596]. In humans, 'catch up growth' (when a low birth weight infant shows an accelerated growth trajectory to 'catch up' with a mean infant body weight) is associated with increased risk of death from coronary heart disease (CHD) in adulthood[597], and an

increased likelihood of excess adiposity in childhood[598]. In my study, female lambs, whose mothers were supplemented with sunflower oil during lactation, were not significantly different in birthweight to other groups, so they exhibited accelerated postnatal growth, rather than true 'catch up' growth. In humans, public health and research initiatives have highlighted the early years as a key period in targeting the prevention of obesity[599-601], and higher omega-6/omega-3 ratios in infant formula compared to breast milk have been named as a potential contributor to the modest protective effect that breastfeeding has against overweight and obesity[602]. There is also an association between greater weight gain in early life and a slight increase in the risk of obesity in adulthood[603]. There is a large body of research which shows convincing evidence that those who are overweight or obese in childhood have a much higher risk of being so in adulthood[18,604], along with an increased risk of obesity related co-morbidities such as metabolic syndrome[605]. A large cohort study (the Harvard Growth Study) followed participants who had been overweight in adolescence (13-18 years) and those who had been lean at this age and found that men who were overweight in adolescence have relative risks of 1.8 for all causes of mortality and 2.3 for mortality from CHD[606]. Interestingly, women did not show an increased risk in these areas, indicating that they may have some protection from CHD. That being said, both men and women had an increased risk of morbidity (as opposed to mortality) from CHD and atherosclerosis if they had been overweight in adolescence.

5.5.3.1 Comparison with Study A (Chapter 3) adiposity and omega-6 fatty acids

Study A found a slight 12% and 13% decrease in milk omega-6 PUFAs and LA (C18:2 n-6) respectively, with canola oil supplementation. It is unclear why these results are different to those found in Study B, which found no difference between milk omega-6 PUFAs or LA between controls and canola supplemented ewes (possible causes of variation in the studies are discussed in Chapter 4, Section 4.6.10). It is, however, interesting that with the decrease in omega-6 PUFAs and LA in Study A, a decrease in pericardial and sternal adipose tissue was observed in female lambs at day 28. Conversely, with no effects of canola oil on omega-6 PUFAs and LA in Study B, an increase in sternal and total adipose tissue was seen in the canola group females at 28 days. This supports my theory that increases in adiposity of the sunflower group in Study B, could be down to the increases seen in milk omega-6 PUFAs and LA. It is interesting that all these effects were only seen in female lambs, reinforcing the theory which I explained in Section 5.5.3, which was that female lambs could have a more efficient conversion of omega-6 fatty acids to endocannabinoids, which may lead to an increase in appetite and thus adiposity.

5.5.4 Effect of altered milk fatty acid profile on lamb's plasma metabolite concentrations

Circulating leptin in later life has been shown to be affected by early life nutrition, with formula fed infants having increased plasma leptin concentrations compared with those who are breastfed[607-609]. Breast milk intake is associated with lower leptin to fat mass ratios than either formula milk intake[608].

The increases I observed in plasma leptin are consistent, to some extent, with those seen in their relative adipose tissue abundance. Theoretically this increase in leptin should reduce the lambs' appetite causing their weight gain to return to a more 'normal' trajectory, in line with control and canola female lambs. The higher plasma leptin concentrations in the female canola group lambs could explain why they showed lower increases in fat mass than the females in the sunflower group. However, when the relationship between plasma leptin and adiposity were analysed, no significance was found, yet the R^2 values were all positive, which suggests that, in keeping with the literature, there is a positive relationship. The reasons for the lack of significance between leptin and fat mass in my study could be due to the limitation that the value for 'total' adipose tissue reported only refers to the total tissue which was sampled (omental, pericardial, perirenal and sternal). If a more precise measure of adiposity had been conducted, such as magnetic resonance imaging (MRI) or dual energy X-ray absorptiometry (DEXA), then I may have seen a significant correlation. The blood sampling was also carried out without controlling the lambs fasting status, which could also have contributed to the lack of significance.

If my previously explained hypothesis is true (female sunflower group lambs having impaired *leptin* signalling due to increased endocannabinoid levels[582]), then their impaired *leptin* signalling is likely to mean that elevated plasma leptin will not be as efficient in decreasing appetite and thus food intake.

The decrease in plasma total cholesterol in the canola and sunflower females, compared to controls at 28 days is not easy to interpret from the analyses here. A limitation of this part of my investigation is that only total cholesterol was measured and therefore, we do not know whether it was HDL or LDL cholesterol, or both which was reduced. This makes it impossible to be certain whether this decrease in total cholesterol is a positive or negative effect. Nevertheless, LDL cholesterol is more abundant than HDL cholesterol[610], therefore, it may be more likely that changes in total cholesterol are due to a decrease, at least in part, of LDL cholesterol. I postulate that the decreases in total plasma cholesterol could be as a

result of a decrease in medium-chain SFAs in the milk of both dietary groups. This would also explain the slightly larger decrease in total cholesterol in the canola females, as SFAs showed a larger decrease in the canola group milk compared to controls. Dietary SFAs, particularly palmitic, lauric and myristic acid (all of which decreased in the milk of canola and, to a lesser extent, sunflower supplemented ewes) have been linked with increasing total cholesterol in humans[611]. Conversely, stearic acid is a long-chain SFA (which was significantly increased in the milk of the canola, and to a slightly lesser extent the sunflower group) that has been shown to reduce total and LDL plasma cholesterol in humans[513], to a similar extent to that seen in my study, so it is possible this could have played a role. Both of these theories fit well with the larger decreases in SFAs associated with increased cholesterol (palmitic, lauric and myristic), larger increase in cholesterol reducing SFAs (stearic acid) in the canola group milk, and the larger decrease in total plasma cholesterol in the canola oil group.

5.6 Conclusion

Firstly, this study found that female lambs whose mothers were supplemented with sunflower oil during lactation showed increased adipose tissue weights and growth rates, although they did not show increased body weights. This could be related to increased omega-6 PUFAs in the milk received by these lambs.

Maternal supplementation of canola oil seemed to delay the decrease in offspring's UCP1, especially in male offspring. Possible causes for this are increased milk *cis*-9, *trans*-11 CLA (in the absence of an increase in *trans*-10, *cis*-12), and increased milk omega-9 erucic acid. The cause of males exhibiting a greater increase in UCP1 is unclear, but could be partly due to some inhibition of *UCP1* by *adiponectin*. Lack of an effect on markers of brite adipose tissue indicated that the increased UCP1 abundance probably results from the retention of BAT due to alterations in the milk FA profile, rather than recruitment of brite adipose tissue.

6 The amount of CLA present in a selection of infant formula milks

Part of my PhD studies involved undertaking an industrial work placement with Danone. I worked at the Danone Nutricia Research and Development Centre in Utrecht, The Netherlands, in the Human Milk Research team. There, I worked to compile evidence for use within the company about CLA and its effects on health, the concentrations and isomer ratios found in breast milk and infant formula and the policy surrounding its inclusion in infant formula, as governed by the EFSA. Danone owns numerous brands of infant formula milk, including Aptamil®, Cow and Gate®, Dumex®, and Milupa®. Part of my work included interpreting data relating to the CLA content of a range of Danone's infant formula milks. These results are the basis for this chapter.

6.1 Introduction

CLA is a collective term for a group of positional and geometric isomers of LA. It was first positively identified by Ha *et al.* in 1987 [293]. The *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers are known to be biologically functional[308] and are the most intensely studied.

CLA has numerous purported health benefits to adults, including inhibiting carcinogenesis[293,295,612], increasing *UCP1* expression[329,331,332], improving bone health[299,613,614], reducing the severity of atherosclerosis[488], and improving glucose tolerance[307]. It also highlighted the paucity of studies into the amount of CLA present in infant formula milk. The presence of CLA in human breast milk has been well established[366,368] and is largely influenced by maternal diet[340,370]. The *cis*-9, *trans*-11 isomer is the most abundant in breast milk, making up around 80-90% of total CLA[366]. It is of interest to formula milk manufacturers to compare CLA concentrations in human and formula milk, as the overall aim is to make the composition of infant formula as similar to breast milk as possible.

The three known studies in relation to CLA in infant formula have all found that it contains lower concentrations than human breast milk[341,368,378]. McGuire *et al.*[368] made direct comparisons between CLA concentrations in 14 samples of breast milk and 16 samples of infant formula milk (from 4 different brands). They found that CLA was detectable in all breast milk samples but in only 37.5% of the formula samples tested. This study was conducted in 1997, and so it is outdated and may not be representative of formula milk currently available. As is the case for a study by Chin *et al.*[341], conducted in 1992. This study tested the CLA content of a variety of foods, including 6 samples of infant formula milk. The found them to contain CLA at 0.03-0.07% of fat, however, did not detect the *cis*-9, *trans*-11

isomer in any of the samples, suggesting that the fat sources in these milks were from vegetable fat, not milk fat.

A more recent study by Oleynik *et al*.[378] conducted in the USA in 2013 compared CLA content of 7 breast milk samples with 4 infant formulas. No CLA was detected in any of the formula milk tested, whereas values ranging from 0.122-0.345% of fat were detected in human milk. This suggests that, currently, CLA content of formula milk is lower than that of breast milk, however, with hundreds of different types of formula milk being available worldwide, the study by Oleynik *et al*. which looks at only 4 types is not likely to be representative. The variation of results between these three studies highlights the fact that more research is needed in this area, as there is a real gap in the literature regarding CLA content in infant formula worldwide.

A potential cause of variation between CLA content of infant formulas is the known seasonal variation of CLA in cow's milk[360,361,363]. Another is, the varying levels of milk fat used in different infant formulas, with some replacing all milk fat with a vegetable fat blend (which contains different proportions of the isomers to human and animal milk fat), and others using varying percentages of milk fat mixed with a vegetable fat blend[276,615-617].

According to common nomenclature, CLA is classed as a *trans* fat. However, TFAs can be further categorised into ruminant and industrial TFAs, of which CLA is the former[495]. Ruminant TFAs have not been linked with the negative health outcomes, such as coronary heart disease and cardiovascular disease associated with industrial TFAs [151-156]. For this reason, ruminant TFAs have been made exempt from legislation set to limit the TFA content of food in numerous countries, including Switzerland, Austria and Iceland[162]. There is currently no EU limitation set on TFA content of food. With the exception of infant formula milk, in which TFAs must not exceed 3% of fat[391], for this limit, there is no recognised difference between ruminant and industrial TFAs.

Chapter 1 details the EU legislation surrounding CLA content in infant formula which, in short, means that CLA cannot be added to infant formula, but that the CLA present within the milk fat can be retained, providing the total *trans* fat content of the milk does not exceed the 3% limit. For this reason, *trans* fat content is routinely measured in Danone's infant formula milk to ensure compliance. Total CLA is measured as part of the TFA measurements, however, specific isomers are not routinely measured.

6.2 Hypothesis

In line with the other studies in this area[341,368,378], I hypothesised that in the infant formula milks analysed, CLA would be not detected or be present, but in lower concentrations than those reported in human breast milk in the literature (approximately 0.27-0.8% of fatty acids)[366,369].

6.3 Methods

A total of 76 Danone infant formula milks were analysed in duplicate, and the mean value taken (with samples showing a variance coefficient >5% between replicates being repeated). The milks were a range of brands and included 42 samples of standard infant formula (IF, marketed for use for infants from 0-6 months of age), 20 samples of follow on formula (FO, marketed for use for infants from 6-12 months of age) and 6 samples of 'growing up' milk (GUM, marketed for use for infants from 1-2 years). The data for the other 8 samples did not specify the age range. All milk analysis was conducted by staff at the Central Laboratory Friedrichsdorf, Germany, using High-performance liquid chromatography (HPLC). My role was to interpret the data.

6.4 Results

6.4.1 Presence of CLA and concentrations of CLA and TFAs

Analysis showed that CLA is present at detectable concentrations in some, but not all, infant formula milks (Figure 6.1). For 25 (33%) of the samples, CLA was undetectable. Implying that there was either a very low concentration of CLA present, below the lower accurate limit of the analysis (0.02%), or that there was no CLA present in the samples. For the purposes of this investigation, as an accurate level could not be assessed, they were classed as containing 0% CLA, as omitting them from the graph entirely would give a false indication that all samples contained CLA. The range of results was wide, with the median being towards the lower end of the range (0.03% of fatty acids). TFAs were detectable in all 76 of the samples and ranged from 0.19% to 2.39% of fat, (Figure 6.1).

As with CLA, there is a large range of results, with the median (0.35% of fatty acids) being at the lower end of the range.

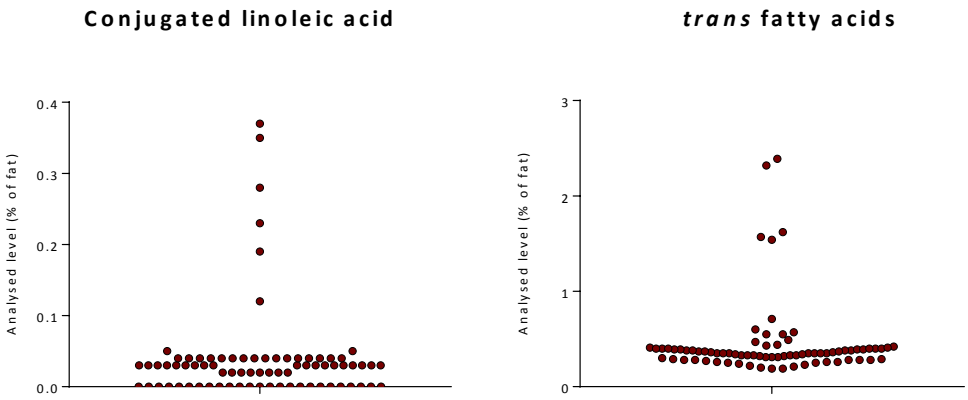

Figure 6.1 Conjugated linoleic acid (CLA) and *trans* fatty acid (TFA) levels (% of fat) in all IFFO milks analysed (n=76 per graph).

6.4.2 Comparison of infant formula milks marketed for different ages

Due to a small number of the samples showing substantially higher CLA content, I investigated whether CLA content would vary with the age group for which they are marketed.

Eight samples which were included in the overall analysis were excluded from the targeted age range-specific analysis due to no age range information being specified in the data.

Figure 6.2 shows that, of the infant formula milks tested, the GUM had significantly more CLA than both the IF (p=0.0012) and FO (p=0.0012) milks. CLA concentrations in IF ranged from 0 - 0.05% of fat. In FO, CLA concentrations were similar to IF, ranging from 0 - 0.04% of fat and CLA in GUM ranged from 0.03-0.35% of fat. Similarly, GUM contained more TFAs than IF (P=0.0008) and FO (P=0.013).

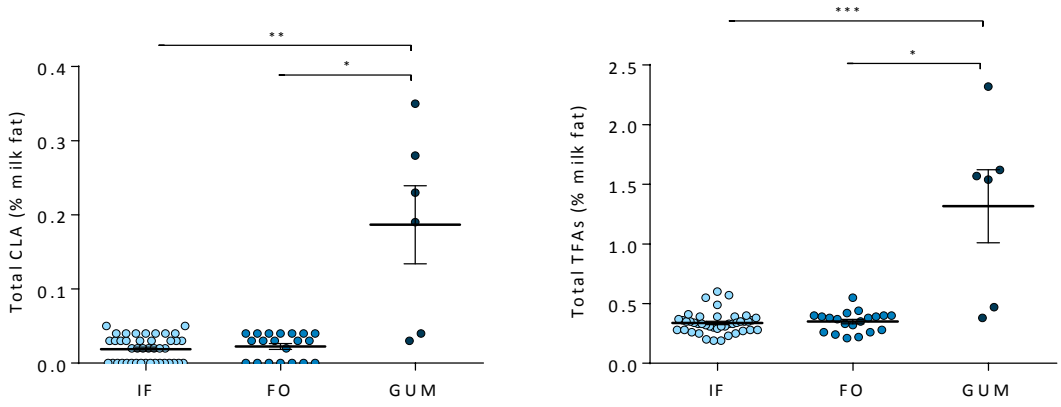

Figure 6.2 Conjugated linoleic acid (CLA) and trans fatty acid (TFA) content (% of fat) by stage of infant milk.

IF=Infant formula (n=42), FO=follow on formula (n=20), GUM=growing up milk (n=6). Values are mean ± SEM. All three stages were compared by Kruskal-Wallis test. Significance is denoted by *p=0.014 and **p=0.0012, (CLA). *p=0.013 and ***p=0.0008 (TFA).

When categorised by intended age group, 60% of IF, 65% of FO and 100% of GUM samples, contained CLA (Figure 6.3).

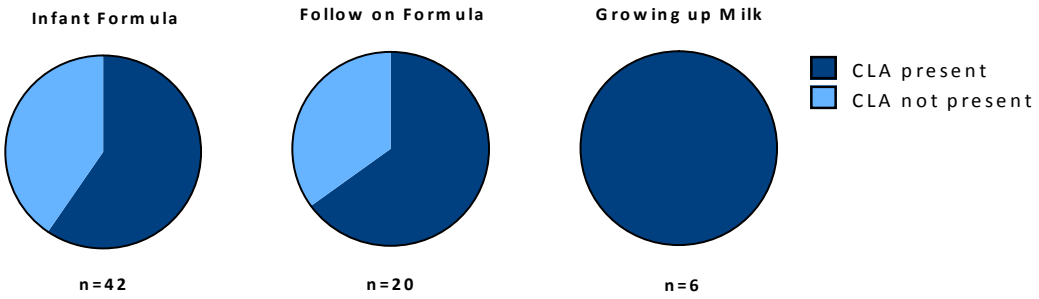

Figure 6.3 Proportion of samples containing conjugated linoleic acid (CLA) categorised by target age group.

Infant formula (0-6 months), follow on formula (6 months-1 year), growing up milk (1-2 years).

6.5 Discussion

6.5.1 CLA was present in some infant formula milks at lower concentrations than reported in human breast milk

Overall, I found that CLA was present in 67% of the milks tested. However, on further investigation, it seemed to vary between the formula milks based on the age group for which they are intended (section 6.5.2).

My results indicate that more formula milks contain more CLA than was suggested by the previous studies[341,368,378], with the most recent study by Oleynik *et al*. in 2013 finding no CLA present in any of the infant formulas tested[378]. However, this study's main limitation was that only 4 brands of infant formula milk were tested and so their sample size was not likely to be representative. A further limitation was that they were all infant formula milks from the USA, and therefore not representative of other countries. The other study, by McGuire *et al.* reported CLA to be present in only 37.5% of the formula samples analysed, with concentrations varying from 0-0.2% of fat[368].

Human breast milk contains approximately 0.27-0.8% of fatty acids as CLA[366,369], but this is very much dependant on maternal diet. Nevertheless, the concentrations in IF (0-0.05% of fatty acids) and FO milk (0-0.04%) from my investigation fall far below those seen in breast milk. The concentrations in GUMs which ranged from 0.03-0.35% of fatty acids, are closer to, but still lower than, those seen in breast milk.

6.5.2 'Growing up' milks contain more CLA acid than other infant milks tested

It is understood that cow's milk can be safely introduced to the diet from 1 year of age[618,619]. GUM is marketed as an alternative to cow's milk for children of 1-2 years of age, and compared with cow's milk, GUM is lower in protein, and has added nutrients including iron, essential fatty acids and vitamins D and E[620]. The WHO recommends breastfeeding until 2 years of age, however, for those carers who are unable/choose not to breastfeed, infant formula milks are an alternative, so for these infants it may be beneficial to have a bridge between FO and cow's milk. Research shows that the risk of α-linolenic acid, iron, vitamin C and vitamin D deficiencies is reduced amongst 1-2 year olds consuming GUM (≥250ml/day) compared to those consuming the same amount of cow's milk[621].

IF and FO are similar in composition, with the most important difference being in their iron content. The minimum and maximum standards for iron are 0.3mg/100kcal and

1.3mg/100kcal respectively for IF and 0.6mg/100kcal and 2.0mg/100kcal respectively for FO[489]. FO milks have a slightly lower minimum fat content (0.4g/100 kcal less) than IF, however, the fat quality and composition criteria are the same[622]. This could explain why I did not see a marked difference between the CLA and TFA content of IF and FO. I did however, observe that the GUMs tested had significantly higher concentrations of both total CLA and TFAs than the IF and FO milks. It is likely that the reason for higher CLA concentrations in GUM is due to it being produced using at least some milk fat. By the age of 6 months, 95% of infants in Europe have been introduced to solids[623], so arguably at this age they will be obtaining CLA from dietary sources such as cheese and yoghurt. This could mean CLA concentrations present in GUM are less necessary than in IF, which is targeted at infants aged 0-6 months. Based on my findings, infants fed IF as their sole source of nutrition will not be receiving as much CLA (if any) as their breastfed counterparts

It is also noteworthy that GUM is a cow's milk alternative, and results from this part of my investigation suggest that CLA concentrations in GUM are lower than those found in cow's milk, which are reported to be around 0.5-1% of fat[363] (GUM 0.03-0.35%). However, it is important to note that a) cow's milk CLA is known to be very variable[361,363], and b) my study only had 6 samples of GUM, so a more comprehensive analysis is required.

The analysis of CLA in the milks tested was not performed by myself. However, as mentioned previously, each sample was analysed in duplicate and the CV between replicated was <5%. Whilst ideally, I would have carried out this research myself, it was not possible due to tight industry regulations. Data should be interpreted with this in mind.

6.5.3 Isomer proportions of CLA in infant formula are not known and are important when considering comparability with human breast milk

When considering the differences between CLA in breast milk and infant formula milks it is not only the total amount of CLA which is important, but also the ratios of the isomers. As mentioned, the majority of CLA in human breast milk is the *cis*-9, *trans*-11 isomer (approximately 80-90%) and the *trans*-10, *cis*-12 isomer makes up only around 1.5% of total CLA[369] with similar amounts present in the milk fat of cows and other ruminants[363]. However, in vegetable fat the ratios are quite different, with *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 isomers each accounting for approximately 40%[341]. With the addition of vegetable fat to formula milk being common, this is a factor to consider. There is evidence to suggest that the two isomers may have different effects[308], with the majority of negative effects being attributed to the *trans*-10, *cis*-12 isomer. Therefore, it is necessary for more

research to be conducted on isomer specific effects in early life and for the ratios of these isomers in infant milks to be considered with as much importance as the total quantity of CLA.

6.5.4 Implications of season on formula milk CLA content

In cow's milk, CLA content is higher in the summer months[360-363] when dairy cows are out to pasture and lower in the winter months when access to fresh grass is limited/non-existent[184]. It is therefore possible that the CLA in infant formula milks which use some milk fat will also show seasonal variation. One potential way to help reduce the effect of season would be to supplement dairy cows in the winter months, when access to fresh grass is limited. The effects seen from my study in Chapter 4 indicate that supplementation of canola oil induced a similar effect on the milk fatty profile to that observed in the summer months, including increased *cis*-9, *trans*-11 CLA, decreased SFAs and increased MUFAs[363]. This shows promise for a potential supplement to ensure the CLA content of milk is maintained all year round.

6.6 Conclusion

I conclude that CLA is present in some, but not all IF and FO milks, yet at a lower level than that reported by studies in human breast milk, supporting my hypothesis. CLA was present in all tested GUM samples, which contained significantly more CLA than IF and FO milk, and was at the lower end of concentrations reported in human breast milk and standard cow's milk. This study did not specifically look at individual CLA isomers, and these need to be further investigated.

7 Summary and conclusions

7.1 Context

In recent decades, there has been an increase in childhood obesity rates in the Western world[4,5], and research shows that individuals who are overweight or obese in childhood have an increased risk of being so in later life[11]. The WHO defines obesity as an excess of adipose tissue[53], yet adipose tissue itself can be divided into further categories.

WAT is a storage site which expands upon positive energy balance, whereas BAT expends energy through the generation of heat, a process mediated by *UCP1*. In sheep[134] and humans[624]. UCP1 peaks around birth and decreases with age. If a way to delay/prevent this decrease could be found, it might aid researchers in developing new ways to prevent/treat obesity.

Early nutrition can affect adult health, including obesity, and UCP1 abundance can be affected by nutrition *in utero*[625], but little work has been carried out into the effects of maternal diet during lactation and its effects on milk composition in the suckling period on offspring's BAT. However, numerous studies have established that breastfeeding offers some protection against obesity[260]. The precise mechanisms for this are unclear, but formula manufacturers strive to make infant formula as compositionally similar as possible to human breast milk.

Studies indicate that concentrations of CLA, a group of isomers of linoleic acid (LA), are lower in infant formula than in human breast milk[368,378]. CLA has shown the potential to increase UCP1[331], though the specific isomers and precise mechanisms behind this effect are not known.

7.2 General aims

My study aimed to investigate whether milk fatty acid profile can affect the development of BAT. In addition, I aimed to investigate the CLA content of a range of infant formula milks, in order to compare their CLA content with those reported for human breast milk.

My main hypothesis was that alterations in the milk fatty acid profile would affect the development of BAT in early life.

7.3 Methods

Sheep that had just given birth were fed 3% of caloric intake as either canola oil or sunflower oil, in order to alter the fatty acid profile of their milk. Offspring were then euthanased at either 7 or 28 days, and their adipose tissue weighed and sampled, to investigate the effect of maternal supplementation on offspring adiposity. Further to this, expression of numerous genes involved with BAT, WAT and metabolism/adipogenesis was measured, along with of the concentration of various plasma metabolites.

Finally, the concentrations of CLA were measured in a range of different infant formula milks, to compare them with the levels reported for human breast milk.

7.4 Findings

7.4.1 Alterations in milk fatty acid profile as a result of maternal canola or sunflower oil supplementation

Previous studies in ruminants have found that supplementation with fatty acids, including canola or sunflower oil, is effective in increasing milk CLA content[406-410]. It also decreases/increases the milk content of SFAs and MUFAs respectively[406-409].

When milk fatty acid profiles from Study A (the feasibility study) were analysed, very few changes were found in canola oil supplemented ewes, in contrast to previously published studies.

Conversely, Study B, in line with previously published studies, found that supplementation of canola and, to a lesser extent, sunflower oil reduced milk SFA content. Both supplements resulted in increased milk MUFAs. Additionally, total CLA and *cis*-9, *trans*-11 content was increased by 28 days to a similar extent in the milk of both the canola and sunflower oil supplemented ewes. In addition, the *trans*-10, *cis*-12 CLA content was increased in the milk of sunflower, but not canola, oil supplemented ewes.

Whilst Study B (Chapter 4) showed relatively wide effects of canola oil supplementation on milk fatty acid profile, Study A (Chapter 3) showed very few changes. The reason for this, despite both studies feeding the same diet to sheep, at the same time of year, is not clear. Different breeds were used for each study, and breed has been shown to affect milk fatty acid profile in sheep[469], so this may well have been a contributory factor. It is also possible that variations in the ewes' pre-study diets, which were not controlled, could have affected milk composition. All ewes were multiparous, although the exact number of previous

births/lactations was not known. Lactation number has been shown to affect milk fatty acid profile in cows[470,474], and so it cannot be ruled out as a potential confounder in my investigations.

Notwithstanding these differences, the results suggest that canola oil supplementation could be a potential way to increase CLA in ruminant milk used to produce infant formula milk, especially in the winter months where milk CLA concentrations are lower[363].

7.4.2 Maternal sunflower oil supplementation results in female offspring with increased adiposity

In Study B, female lambs whose mothers were supplemented with sunflower oil showed increased growth rates and adipose tissue weights. This could potentially be linked to the omega-6 (LA) content of the milk, which was raised in the sunflower group. LA and its derivative arachidonic acid have been associated with increased adiposity in rodents[286]. Further support for this comes from my findings in Study A, which showed a decrease in milk LA content with maternal canola supplementation, accompanied by a decrease in adipose tissue depot weights of female lambs.

The gender effect could be as a result of increased endocannabinoid receptors in females[586]. Endocannabinoid activity is increased by omega-6 fatty acids[581], and can lead to hyperphagia[582] and increased lipogenesis and adiposity[581,583]. These effects would be more pronounced in females if they possessed more endocannabinoid receptors than males, which appears to be the case in early life in rodents[586]. However, this does not explain the gender specific effects in Study A, making it likely that other mechanisms were also involved.

7.4.3 Maternal canola oil supplementation prevents/delays the loss of UCP1 in male and, to a lesser extent, female offspring

Perirenal adipose tissue depots of lambs fed by mothers supplemented with canola oil showed areas of multilocular brown adipocytes, which stained positively for UCP1 at 28 days of age. This is in contrast with the control and sunflower lambs, whose perirenal adipose tissue was white and showed little evidence of UCP1 at this age. The male lambs in the canola group had more areas of brown adipocytes stained for UCP1 than the females, yet both genders showed no significant decrease of UCP1 between 7 and 28 days, compared with control and sunflower group lambs, who displayed a significant decline of UCP1 between these time points.

The precise cause of this effect is unknown, but there is potential that the increase in *cis*-9, *trans*-11 CLA, coupled with no change in *trans*-10, *cis*-12 CLA (which was increased in the sunflower group), could have modulated this effect. Another possibility is that the greater decrease in milk medium-chain SFAs with canola, compared with sunflower, supplementation could be involved in the effects seen in *UCP1*. The reason for males exhibiting a stronger effect is also unclear. It may be related to the increases in *adiponectin* mRNA which were seen in the females only at 7 days, as *adiponectin* inhibits *UCP1*[102], and therefore may have partially counteracted the mechanism by which UCP1 was retained.

7.4.4 Infant formula milk tested contained less CLA than that reported in human breast milk

Of the 76 infant formula milks tested, 67% contained CLA, which is more than previous studies have suggested[341,368,378]. The formula milks were categorised by their target age group: infant formula (IF) for 0-6 months, follow on formula (FO) for 6-12 months, and 'growing up' milk (GUM), for 1-2 years. I found that GUM contained significantly more CLA than either IF or FO milk. IF and FO contained very low concentrations of CLA compared with values previously reported in human breast milk. From this investigation, my results suggest that infants fed with formula milk will be receiving less CLA than their breastfed counterparts and, despite GUM having the highest CLA of the milks analysed, 1-2 year-olds consuming GUM (marketed as an alternative to cows' milk) are likely to be receiving less CLA than those consuming cows' milk.

7.5 Limitations of investigation

7.5.1 Animal model

Whilst sheep have advantages as an animal model, as discussed in Chapter 1, they also have limitations. For nutritional studies, sheep have the obvious disadvantage of being herbivores and ruminants, and, therefore, metabolise energy differently to humans. This means that the intervention used in my investigation cannot be simply translated to humans by, for example, supplementing the diet of lactating women with canola/sunflower oil. However, this limitation is less of a concern with the nutrition received by newborn lambs, as their rumen development is not complete at this stage, and its development is dictated by the introduction of solid food[626]. This does not usually occur until ~6-8 weeks of age in lambs[627], and the lambs studied here were mother fed for the entire duration (up to 28 days) of my study. Furthermore, the suckling movement initiates the so-called

'oesophageal groove reflex', which results in milk travelling straight to the abomasum from the oesophagus, bypassing the rumen, reticulum and omasum[628].

Another limitation of sheep is the fetal number. Some ewes gave birth to twins and others triplets, whereas human mothers usually give birth to a singleton. Fetal number is known to affect birth weight[629] although, there were no significant differences in mean birth weight between dietary groups in my investigations. Fetal number has also been found to affect later fat mass[630], and is, therefore, a potential confounder in my investigation. This limitation was partially rectified postnatally by immediately removing a lamb from ewes that gave birth to more than two lambs, resulting in each ewe raising two lambs. Another potential confounder is that until 7 days, each ewe raised two lambs and after this point (the first lamb being euthanased) each ewe raised one lamb. Meaning this should be considered if directly comparing the two time points. In my investigation, day 7 and day 28 lambs within groups have not been directly compared, except in the case of UCP1 decreasing with age (Chapter 5, Figure 5.21). This comparison alone may be flawed due to this limitation, however, it was not the decrease itself which I was interested in, but the lack of a decrease in the canola group, compared with the other two groups (who were also exposed to this limitation).

The fact that newborn lambs are quadrupeds and able to walk at birth means that they are able to be more physically active in early life compared to human infants. In addition, lambs are born with wool which will aid them in maintaining body temperature. However, in modern times, human neonates are generally born in heated buildings and clothed shortly after birth. Nonetheless, these differences should be considered when translating research in the BAT of lambs to humans.

The sheep genome sequence only became available in its entirety in 2014[631]. This, coupled with the relatively few studies published using sheep as a model in BAT research, means that commercially available antibodies and published primer sequences are limited, which restricted the number of genes available for analysis.

7.5.2 Multiple effects of maternal supplementation on milk fatty acid profile

The changes in milk fatty acid profile as a result of canola and sunflower oil supplementation were relatively wide-ranging in Study B. This means that it is not possible to attribute the effects on offspring to any single fatty acid. In order to do this, it would have been ideal to supplement ewes/lambs directly with a specific fatty acid. At the time

the studies were conducted, however, individual CLA isomer supplements were not readily available in a rumen-protected form. As no previous work has been carried out relating to milk fatty acid profile and BAT in larger mammals, it was most appropriate to alter the milk fatty acid profile through fatty acid supplementation, which would be sufficient to test whether this could affect BAT, and the results could inform further investigation.

It is also possible that supplementation altered other aspects of the milk's nutritional composition, such as protein, which were not measured in my investigation. It has been reported that dietary fat in cows can suppress milk protein content[632], but this was accompanied by an increase in milk fat, which was not found in my study. However, the possibility of supplementation affecting other aspects of the milk composition cannot be ruled out.

7.5.3 Fat supplements

The fat administered to ewes in my investigations was supplementary and, therefore, the ewes receiving each supplement received 6% of calories from fat, as opposed to 3% in controls. Therefore, the supplemented ewes were receiving approximately 400 more kilocalories than control ewes, per day. This means it is possible that results could be due to increased dietary fat, or increased in energy intake, as well as the type of fat. However, the different effects observed in milk fatty acids between the two supplemented groups (who received the same percentage of fat) suggest that the type of fat was responsible. In addition to this, the fat content of milk produced by the ewes was not significantly affected by either supplement, so the effects on offspring were probably not attributable to milk total fat content, but more likely the individual fatty acid concentrations.

7.5.4 Western blotting

Unfortunately, the sample numbers for Western blotting were lower than those for other analyses. This was due to low yield from mitochondrial preparations which, due to time constraints and insufficient remaining tissue samples could not be repeated. Therefore, there was a low sample size of female lambs at 28 days and this lack of power is likely to be the reason for the lack of statistical significance.

7.5.5 CLA analysis of infant formula milk

Only total CLA measurements were available for interpretation, not specific isomers. It is known that *cis*-9, *trans*-11 is the major isomer in human breast milk[366] and ruminant milk[363].

However, in infant formula milk the proportions of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers are likely to be different, due to vegetable oil, which contains approximately a 50:50 ratio of *cis*-9, *trans*-11 to *trans*-10, *cis*-12[341], being added to many infant formula milks[277]. The two isomers have opposing effects on blood lipids in adults[308], and the *trans*-10, *cis*-12 isomer has been associated with several negative health outcomes, including hyperinsulinaemia[309], insulin resistance[309,310] and liver steatosis[309]. The limited number of previous studies of infant formula have reported very different concentrations of individual isomers, one finding the majority of CLA to be *cis*-9, *trans*-11, and the other detecting CLA, yet no *cis*-9, *trans*-11[341,368]. Therefore, with the aim of making infant formula as compositionally similar to human breast milk as possible, further research into the quantities of specific CLA isomers is vital.

7.6 Future work

7.6.1 Effects beyond 28 days

Although the mean protein abundance of UCP1 was not significantly lower at 28 days, compared to 7 days, in those lambs whose mothers were supplemented with canola oil during lactation, they were numerically lower. It would be useful to investigate this over a longer time period, to see if the maternal canola oil supplementation prevented the offspring's loss of UCP1, or simply delayed it. It would be particularly informative to investigate whether, if this study was carried out over a longer time period and UCP1 was indeed retained, the offspring exhibited any phenotypic changes (i.e. lower body weight or resistance to obesity), potentially as a result of the retained UCP1.

7.6.2 Direct supplementation with fatty acids

As discussed above, in order to investigate the potential that changes in certain fatty acids were involved with the retention of UCP1 at 28 days in my investigation, it would be beneficial to supplement ewes/lambs directly with specific fatty acids. For example, individual CLA isomers, or SFAs, to test whether they enhance or inhibit UCP1 abundance.

7.6.3 Increasing CLA in infant formula milk

My results from Chapter 4 showed that canola oil successfully increased the *cis*-9, *trans*-11 content in milk. This supplementation could potentially be of use to maintain milk CLA content in dairy cows, whose milk is used to produce infant formula milk. The CLA content of milk is known to be lower in the winter months[363]. Previous studies supplementing

canola and sunflower oil suggest that a similar effect on milk fatty acid profile is seen in cows[407,408] to that which I observed in sheep. The increase in milk CLA content by dietary supplementation would be allowed under the EFSA regulations, which prohibit the addition of CLA to infant formula but permit that which is present in the milk fat to remain, providing that the 3% *trans* fatty acid limit is not exceeded[489]. Increasing milk CLA in this way would also rely on at least some of the milk fat being retained during the formulation of the infant formula milk, and not completely replaced with vegetable fat.

7.7 Final remarks

This study has demonstrated that there is potential for the loss of UCP1 in early life to be delayed/prevented by altering the composition of milk fed to offspring. In addition to this, it has found that the effects of milk fatty acid profile on *UCP1* are modulated, at least to some extent, by gender. These findings will inform future research that is targeted at making infant formula milk more comparable to human breast milk, with a view to reducing the disparity in obesity risk between breastfed and formula fed infants.

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Appendix 1: List of suppliers

ABgene	Abgene House, Blenheim Road, Epsom, KT19 9AP, UK. www.abgene.com
Ambion	850 Lincoln Centre Drive, Foster City, CA 94404, USA. www.ambion.com
Anglia Scientific	94 Fordham Road, Soham, Ely, Cambridgeshire, CB7 5AJ, UK. www.angliainst.co.uk
Applied Biosystems	850 Lincoln Centre Drive, Foster City, CA, 94404, USA. www.appliedbiosystems.com
BD	Becton Dickinson and Company**,** Belliver Industrial Estate, Belliver Way, Plymouth, Devon PL6 7BP, UK. www.bd.com
Dako	Cambridge House, St Thomas Place, Ely, Cambridgeshire, CB7 4EX, UK. www.dako.com
GE Healthcare	Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK. www.gehealthcare.co.uk
GraphPad Software	7825 Fay Avenue, Suite 230, La Jolla, CA, USA. www.graphpad.com
Greiner Bio-One	Brunel Way, Stroudwater Business Park, Stonehouse, GL10 3SX, UK. www.greinerbioone.com
Hoefer Inc. MA	84 October Hill Rd, Suite 10, Holliston, 01746, USA. www.hoeferinc.com
Leica Biosystems	Balliol Business Park West, Newcastle Upon Tyne, NE12 8EW, UK. www.leicabiosystems.com
Leica Microsystems	Larch House, Woodland Business Park, Breckland, Linford Woods, Milton Keynes, MK14 6FG, UK. www.leica-microsystems.com
Manor Farm Feeds	Green Lane, Owston, Oakham, Leicestershire LE15 8DH, UK. www.manorfarmfeeds.co.uk
Media Cybernetics	401 N Washington Street, Rockville, 20850, MD, USA. www.mediacy.com
Menzel-Gläser	Glasbearbeitungswerk GmbH & Co., Saarbrückener Str. 248, D-38116, Braunschweig, Germany. www.menzel.de
Millipore	Croxley Green Business Park, Watford, Hertfordshire, WD18 8YH, UK.

	www.emdmillipore.com
Primer Design	Millbrook Technology Campus, Second Avenue, Southampton, Hampshire, SO15 0DJ, UK.
	www.primerdesign.co.uk
Promega	Enterprise Road, Southampton, SO16 7NS, UK.
	www.promega.com
Qiagen	Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK.
	www.qiagen.com
QImaging, Surrey	19535 56th Avenue, Surrey, BC V3S 6K3, Canada.
	www.qimaging.com
Randox Laboratories	55 Diamond Road, Crumlin, County Antrim, BT29 4QY, UK.
	www.randox.com
Raytek	26 Norton Park View, Sheffield, S8 8GS, UK.
	www.raytek.co.uk
Sakura Finetek	Flemingweg 10A, 2408 AV Alphen aan den Rijn, Netherlands.
	www.sakura.eu
Sarstedt	Sarstrabe 1, 51588 Nümbrecht, Germany.
	www.sarstedt.com
Scientific Laboratory Supplies	Orchard House, The Square, Hessle, East Riding of Yorkshire, HU13 0AE, UK.
	www.scientificlabs.co.uk
Sigma-Aldrich Company	The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK.
	www.sigma-aldrich.com
Simport Scientific	2588 Bernard-Pilon Street, Beloeil, QC J3G 4S5, Canada.
	www.simport.com
Thermo Scientific	Sarstrabe 1, 51588 Nümbrecht, Germany.
	www.sarstedt.com
Thermo Shandon	93-96 Chadwick Road, Runcorn, Cheshire, WA7 1PR, UK.
	www.thermofisher.com
Thistle Scientific	DFDS House, Goldie Road, Uddingston Glasgow G71 6NZ.
	www.thistlescientific.co.uk
TouchGene Gradient, Techne	Hinxton Road, Duxford, Cambridgshire, CB2 4PZ, UK.
	www.techne.com
VWR	Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, UK.
	www.vwr.com

Appendix 2: List of infant formula milk brands

Brand	Type
Milupa Milumil 2 Folgemilch	FO
Aptamil 2 800 Exp.	FO
Mellin 2	FO
Cow + Gate 2 follow on milk	FO
Nutrilon 2	FO
BASE FOLLOW ON LCP 0.3:0.3 FK 50:50	FO
Dumex Gold Precinutri 2 - DG2	FO
BASE FOLLOW ON 2 FK 80:20	FO
Malyutka 2	FO
BASE FOLLOW ON AR LCP 4 k 80:20	FO
Dumex 2 Precinutri	FO
Malyutka 2	FO
Karicare 2 - Growing Baby	FO
Karicare 2 - Growing Baby	FO
Aptamil 2	FO
Dumex Dupro 2 Follow on Formula	FO
Follow on formula base powder	FO
Aptamil Gold + 2	FO
Dexolac Premium 3	FO
FO follow on Base 120	FO
IP PWD base powder GUM1 F	GUM
Dumex Precinutri 3 - DG3	GUM
Instant growing up milk powder	GUM
Nutrilon Premium 3	GUM
BP base powder GUM1 LCP7 MF	GUM
Dumex 3 Precinutri	GUM
Dumex 1 Precinutri	IF
Dumex 1 Precinutri	IF
Dumex 1 Precinutri	IF
Dumex Gold Precinutri 1 - DG1	IF
Gallia dès la naissance 1	IF

Bebelac AR from birth onwards	IF
Milupa Milumil 1	IF
Aptamil Hungry Milk from birth	IF
Aptamil 1 first milk ready to feed	IF
Aptamil 1	IF
Nutrilon 1	IF
Nusobee Soya	IF
Karicare Plus 1 from birth	IF
Milupa Milumil HA 1	IF
Nutrilon Premium 1	IF
Bebelac FL infant milk free of lactose from birth onwards	IF
Aptamil SL Milchfrei	IF
Aptamil Pro futura 1	IF
Bebelac Premature	IF
Bebelac 1 Infant milk formula from birth onwards	IF
Base Bebelac FL	IF
Aptamil 1	IF
Milupa Aptamil Prematil Spezialnahrung	IF
Gallia Calisma 1	IF
Milumil 1	IF
Malyutka 1 from birth	IF
Bebelac 1	IF
Aptamil Pepti Junior	IF
Malyutka 1 from birth	IF
Nutribaby 1 formula bayi	IF
Milupa Aptamil 1 Infant formula	IF
Dumex Dulac 1 Infant Formula	IF
Cow + Gate Happy baby 1	IF
Malyutka 1 from birth	IF
Bebilon Pepti	IF
Bebelove 1 formula bayi	IF
Malysh 1	IF
Infant formula (HiQ Super Gold Synbio ProteQ (TM))	IF

Aptamil Gold Plus Pronutra 1	IF
Cow + Gate infant milk for hungrier babies from newborn	IF
Milupa Aptamil HA 1	IF
Base Start LCP 4FK	IF
Base start extra LCP 0.35:0.32 Fk	Unknown
IP PWD BP 40-60 LCP5	Unknown
Milupa Aptamil HA 1	Unknown
Milupa Aptamil HA 2	Unknown
Base Start LCP 4FK	Unknown
Aptamil Prematil HA	Unknown
Milupa Milumil 2+	Unknown
BP base powder MF	Unknown