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# Nutritional Programming of Brown Adipose Tissue

By

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## Abstract

Maintenance of functional brown adipose tissue (BAT) beyond the newborn period, through its role in expending energy in thermogenesis, provides a potential target to prevent childhood obesity. As the body's fat distribution can be programmed by nutrition in early life, this study investigated whether modifying the maternal diet in the immediate newborn period increases the thermogenic potential of major brown fat depots. In humans and sheep, UCP1 decreases after birth, thus, BAT gradually transitions to be white adipose tissue (WAT). Three major adipose tissue depots in sheep (sternal, perirenal and epicardial) were studied which are all populated with brown adipocytes in early postnatal life, and then undergo a transition to WAT by the end of first month of life. Heat production in BAT is mediated by uncoupling protein (UCP)1 and fatty acids have the potential to increase the amount of UCP1. The aim of this thesis was, therefore, to determine whether maternal fatty acid supplementation with a readily available short-chain fatty acid (i.e. canola or sunflower oil) modifies milk fatty acid profile and thus the development of adipose tissue in the offspring.

Ewes, that each reared twin lambs, were fed a control diet or one supplemented with either 3% canola or sunflower oil from the day of delivery throughout lactation. Milk samples, ewe and lamb weights were taken at 7 and 28 days and offspring underwent tissue and blood sampling at either 7 or 28 days of age. The mRNA expression of regulatory genes associated with thermogenesis and fatty acid metabolism was measured. The abundance of UCP1 and other mitochondrial proteins was determined by immunohistochemistry and immunoblotting. Finally, microarray datasets were subjected to multi-region bioinformatics analysis in order to obtain biologically meaningful information about the examined adipose tissue depots.

Supplementation of the maternal diet with canola oil, and to lesser extent, sunflower oil, resulted in decreased medium-chain saturated fatty acids and increased monounsaturated fatty acids content of milk. Omega-6 polyunsaturated fatty acids was also increased in milk of ewes that received the sunflower oil supplement.

Although maternal fatty acid supplementation did not alter the mRNA expression of UCP1 in suckling lambs, tissue morphology (histology and microscopy) and protein abundance showed an increased UCP1 in adipose tissue located in the perirenal at 28 days of age compared with controls, suggesting canola oil delayed the rate of BAT loss. Microarray analysis indicate that each depot exhibited a distinct profile of gene expression and contained a small number of unique modules of co-expressed genes associated with distinct biological processes. Compared to other adipose tissue depots, perirenal showed a greater capability to respond to changes in the maternal diet. It was the most responsive adipose depot to maternal supplementation with canola oil causing an increase in the expression of some genes associated with the modulation of thermogenesis in BAT. With regards to genes involved in fatty acid metabolism, the epicardial depot appeared to be the primary site of unsaturated fatty acid synthesis in the first week of age, whilst this was dominant in the perirenal depot by day 28 of age when BAT is mostly converted into WAT.

In conclusion, manipulating the fatty acid profile of milk ingested by the newborn may delay, or even prevent, UCP1 loss in early life. In addition, the recognition of the different characteristics of adipose tissue depots in early life expands the current understanding of adipose tissue development and the variations of their response to dietary intervention.

## Acknowledgements

This thesis is the culmination of my PhD journey. Although only my name appears on the cover of this dissertation, a great many people including my family, my supervisors, my friends and my colleagues have contributed to this huge task.

Foremost, I thank God for giving me the strength and patience to work through all these years so that today I stand proudly with my head held high.

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To all who have contributed to this journey in one way or another: ''I can no other answer make, but, thanks, and thanks'' - Shakespeare, cited in Craig (1914).

# 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 November 2013 and April 2018.

Unless otherwise stated, this thesis illustrates my own work completed under the supervision of Professor Michael Symonds, Professor Helen Budge of the University of Nottingham.

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

Reham Ibrahim Alagal

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# Presentations

## Oral presentations

**Alagal, R.**; Budge, H. and Symonds, M. Fight fat with fat. M&HS Faculty Postgraduate Research Forum, School of Medicine, University of Nottingham. June 2015.

**Alagal, R.**; Budge, H. and Symonds, M. Mitochondrial protein abundance in adipose tissue of postnatal sheep. Research group away day, Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham. July 2015.

**Alagal, R.**; Woods, R.; Budge, H. and Symonds, M. Mitochondrial protein abundance in adipose tissue of postnatal sheep and nutritional modulation. Sue Watson Postgraduate Presentation Prize, School of Medicine, University of Nottingham. October 2015.

**Alagal, R.**; Budge, H. and Symonds, M. Immunohistochemistry quantification using Whole Slide Imaging (WSI) Technology. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham. September 2016.

**Alagal, R.**; Woods, R.; Fainberg, H.; Budge, H. and Symonds, M. The effects of supplementing ewe diet during lactation with fatty acids on milk fatty acid profile. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham. November 2016.

**Alagal, R.**; Fainberg, H.; Woods, R.; Budge, H. and Symonds, M. The effects of supplementing the maternal diet during lactation on offspring's transcriptional landscape of the major brown adipose depots during early life. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham. June 2017.

**Alagal, R.**; Budge, H. and Symonds, M. The effects of manipulating dietary fatty acid supplementation on the expression profiles of genes associated with fatty acid

metabolism in brown adipose tissue depots. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham, February 2018.

### Poster presentations

**Alagal, R.**; Budge, H. and Symonds, M. Fight fat with fat. M&HS Faculty Postgraduate Research Forum, School of Medicine, University of Nottingham. June 2015.

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**Alagal, R.**; Woods, R.; Birtwistle, M.; Budge, H. and Symonds, M. Fatty acids supplementation increase the abundance of mitochondrial protein in adipose tissue of postnatal sheep. The Fourth Wonca EMR Family Medicine Congress, Abu-Dhabi, United Arab Emirates. 2-4 March 2017.

**Alagal, R.**; Birtwistle, M.; Davies, G.; Woods, R.; Budge, H. and Symonds, M. The effect of supplementing the mother's diet during lactation on their milk's fatty acid profile and offspring adipose tissue. Nottingham Paediatric Research Showcase, Nottingham, United Kingdom. June 2017.

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# List of abbreviations

| Abbreviation | Definition |
|---|---|
| AA | Arachidonic acid |
| aBAT | Axillary brown depot |
| AC | Adenylyl cyclase |
| ACC | Acetyl-coenzyme a carboxylase |
| ACOT11 | Acyl-coenzyme A thioesterase 11 |
| $AL_3^+$ | Aluminium ions |
| ALA | α-linolenic acid |
| APS | Ammonium persulphate |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| BAT | Brown adipose tissue |
| BBB | Blood–brain barrier |
| BCA | Bicinchoninic acid |
| BH | Biohydrogenation |
| BLAST | Basic Local Alignment Search Tool |
| BMI | Body Mass Index |
| BMR | Basal metabolic rate |
| bp | Base pair |
| BRITE | Brown in white adipocytes |
| BSA | Bovine serum albumin |
| C | Catalytic subunits |
| cAMP | Cyclic adenosine monophosphate |
| cBAT | Cervical brown depot |
| cDNA | Complementary deoxyribonucleic acid |
| $C_f$ | Final concentration |
| $C_i$ | Initial concentration |
| CLA | Conjugated linoleic acid |
| COX1 | Cytochrome c oxidase 1 |
| COX2 | Cytochrome c oxidase 2 |
| Cp | Crossing point |
| CRAN | Comprehensive R Archive Network |
| Ct | Cycle threshold |
| CVD | Cardiovascular disease |
| DAB | 3,3’-diaminobenzidine |
| DGLA | Dihomo-gamma-linolenic acid |
| DHA | Docosahexaenoic acid |

| | |
|---|---|
| DIT | Diet-induced thermogenesis |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleoside triphosphates |
| DOHaD | Developmental Origins of Health and Disease |
| E | Efficiency of the reaction |
| EAT | Epicardial adipose tissue |
| EDTA | Ethylenediaminetetraacetic acid |
| EFAs | Essential fatty acids |
| EFSA | European Food Safety Authority |
| ELOV3 | Elongation of very long chain fatty acids 3 |
| EPA | Eicosapentaenoic acid |
| eWAT | Epididymal adipose depot |
| FABP | Fatty acids binding proteins |
| FABP3 | Fatty acid binding protein 3 |
| FADS2 | Fatty acid desaturase 2 |
| FAS | fatty acid synthetase |
| FAs | Fatty acids |
| FDG | 18 F-fludeoxyglucose |
| FDR | False discovery rate |
| FFAs | Free fatty acids |
| GC | Guanine/cytosine |
| gDNA | Genomic deoxyribonucleic acid |
| GWAS | Genome-wide association studies |
| H&E | Haematoxylin and Eosin |
| HIRE | Heat-induced epitope retrieval |
| HOXC9 | Homeobox C9 |
| HRP | Horseradish peroxidase |
| HSCIC | Health and Social Care Information Centre |
| HSE | Health Survey for England |
| HSL | Hormone sensitive lipase |
| iBAT | Interscapular brown depot |
| IGF-1 | Insulin-like growth factor 1 |
| IHC | Immunohistochemistry |
| LA | linoleic acid |
| LCFAs | Long-chain fatty acids |
| LCPUFAs | Long-chain polyunsaturated fatty acids |
| LEAR | Low erucic acid rapeseed |
| LPL | Lipoprotein lipase |
| M | Stability value |

| | |
|---|---|
| mBAT | Mediastinal brown depot |
| MCFAs | Medium-chain fatty acids |
| MG | Monoacylglycerol |
| ML | Machine learning |
| MSCs | Mesenchymal stem cells |
| MUFAs | Monounsaturated fatty acids |
| mWAT | Mesenteric adipose depot |
| MYF5- | Myogenic factor 5- progenitors |
| MYF5+ | Myogenic factor 5+ progenitors |
| NASC | Nottingham Arabidopsis Stock Centre |
| NCBI | National Center for Biotechnology Information |
| NE | Norepinephrine |
| NEFAs | Non-esterified fatty acids |
| NICE | National Institute for Clinical Excellence |
| NRTC | No-reverse transcriptase control |
| NST | Nonshivering thermogenesis |
| NTC | No-template control |
| PAT | Perirenal adipose tissue |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PET | Positron emission tomography |
| PG | prostaglandins |
| PIC | Preinitiation complex |
| PKA | Protein kinase |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| prBAT | Perirenal adipose depot |
| PRDM16 | PR domain containing 16, |
| PRLR | Prolactin receptor |
| PSOL | Positive sample-only learning |
| PUFAs | Polyunsaturated fatty acids |
| QC | Quality control |
| qPCR | Quantitative polymerase chain reaction |
| R | Regulatory subunits |
| $R^2$ | Correlation coefficient |
| RF | Random forest |
| RIN | RNA integrity number |
| RIP140 | Receptor-interacting protein 140 |
| $RT^-$ | Non-reverse transcription enzyme control |
| $RT^+$ | Reverse transcription enzyme control |

| | |
|---|---|
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| SCD1 | Stearoyl-CoA desaturase-1 |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| SFAs | Saturated fatty acids |
| SHFAs | Short-chain fatty acids |
| SHOX2 | Short stature homeobox 2 |
| SNS | Sympathetic nervous system |
| TEMED | Tetramethylethylenediamine |
| TFAs | *trans* fatty acids |
| TGs | Triglycerides |
| Tris | Tris(hydroxymethyl)aminomethane |
| TRPs | Transient receptor potential channels |
| TTBS | Tris-buffered saline with Tween® 20 |
| UCP1 | Uncoupling Protein 1 |
| USFAs | Unsaturated fatty acids |
| VDAC | Voltage-dependent anion channels |
| $V_f$ | Final volume |
| $V_i$ | Initial volume |
| VLDL | Very low-density proteins |
| WAT | White adipose tissue |
| WGNCA | Weighted correlation network analysis |
| WHO | World Health Organization |
| WOF | World Obesity Federation |
| ω-3 | Omega-3 fatty acids |
| ω-6 | Omega-6 fatty acids |

# Chapter 1. Introduction

## 1.1 Obesity

The prevalence of 'overweight and obesity' is a medical problem affecting all age groups in nations worldwide. Although the root causes of obesity and the reasons behind its steady increase are still controversial issues, the negative effect on human health is undisputed.

### 1.1.1 Definitions of Overweight and Obesity

Definitions of 'overweight' and 'obesity' vary among organisations and by age group. An adult is viewed as overweight when his or her Body Mass Index (BMI) ranges between (25.0-29.9) kgm-2 and obese when the BMI exceeds 30.0 kgm-2, as reported by World Health Organisation (WHO) (WHO, 2008).

In contrast, BMI in children changes dramatically with age. For example, the average BMI is low at birth (13 kg/m²) and increases in the first year of life to 17 kg/m² before falling to 15.5 kg/m² by the sixth year. By age 20, the BMI has risen again, to 21 kg/m². There is a clear need to identify BMI benchmarks as related to age when diagnosing childhood obesity (Barlow & Dietz, 1998). For example, the National Institute for Clinical Excellence (NICE) uses the 1990 UK BMI chart (Freeman et al., 1995), which compares the weight and height of overweight and obese children with the average BMI of healthy children in the same age group. Based on this, a BMI ≥ 91st population centile and < 98th centile indicates overweight, and values > 98th centile indicate obesity (NICE, 2006). In the United States, based on nationally representative survey data, the 85th and 95th centiles of BMI indicate overweight and obesity, respectively (Cole et al., 2000).

### 1.1.2 Overweight and Obesity: A Global Problem

Overweight and obesity is becoming a world-wide phenomenon that are not confined to a particular community or age group. Overweight and obesity may occur at different stages of life, regardless of age or socioeconomic status. Studies indicate that the obesity problem continues to increase in many countries such as Germany (European Commission, 2010), Brazil (Seki et al., 2009), India (Goyal et al., 2010) and China (Shen et al., 2012). Figure 1.1 shows the prevalence of overweight and obesity around the word.

In England, Health and Social Care Information Centre the reported that the ratio of obese adults increased from 13.20% to 24.40% of men and from 16.40% to 25.10% of women in 1993 to 2012 (HSCIC, 2014). Future projections indicate a significant increase in obesity rates reaching 47% of males and 36% females by 2035, with the rates expected to exceed half of the population in 2050 (Foresight, 2007). This steady rise in obesity rates is not limited to England, but is spread all over the world. In 2008, it was estimated that a quarter of the population of the world, approximately 1.5 billion people, were overweight and obese (WHO, 2011).

Rates of obesity among children have been increasing in England during the past two decades, with a slight decrease in very recent years (HSCIC, 2014). According to Health Survey for England (HSE) statistics, the proportion of obese children between the ages of 2 and 15 increased from 11% among boys in 1995 to 18% in 2004/5 and from 12% among girls in 1995 to 19% in 2004/5. This was followed by a small decline in 2011, when rates reached 17% and 16% among boys and girls, respectively, before settling at 14% for both sexes in 2012 (HSCIC, 2014).

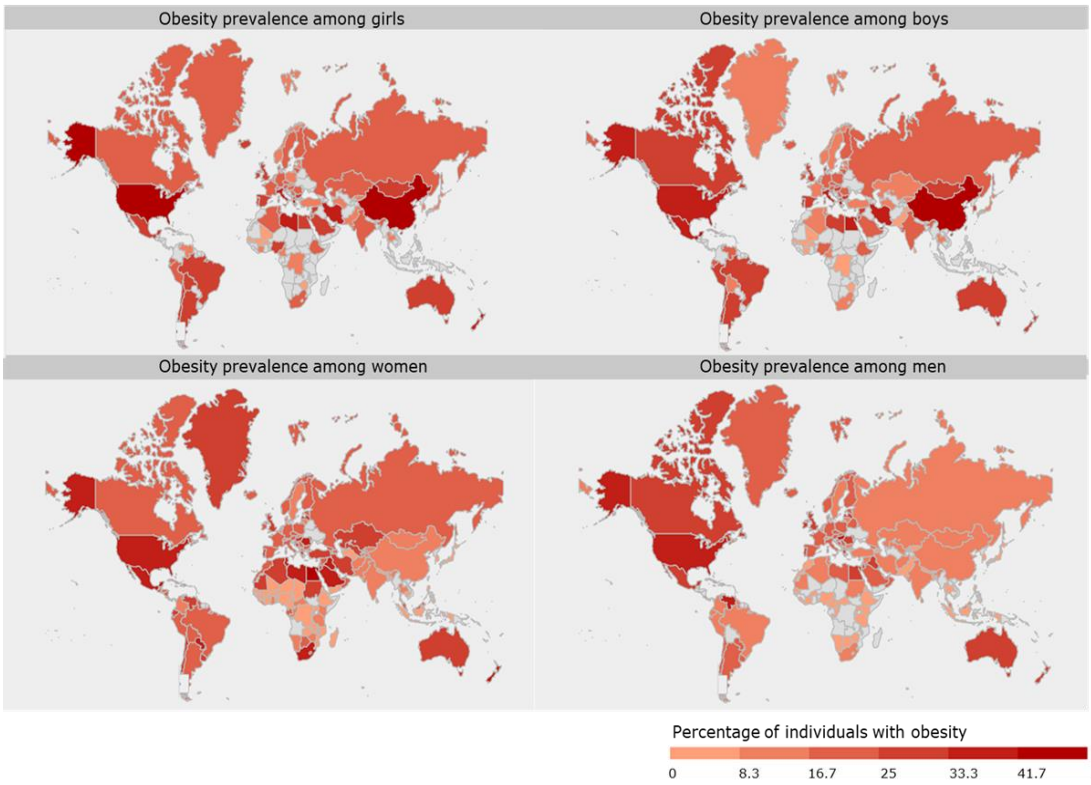



**Figure 1.1 The global distribution of obesity.**

Data from World Obesity Federation (WOF) collected from over 50 countries worldwide according to the last national update. Gray indicates no obesity statistics available.

### 1.1.3 Causes and Treatment

The cause of overweight and obesity can be summarised as an accumulation of triglycerides (TGs) and lipids in adipose tissue due to long-term positive energy balance. The rapid spread and high rates of obesity cannot be attributed to genetic factors alone (Symonds et al., 2011), as many other factors are involved. For example, there are etiological, environmental and social factors; psychological conditions, dietary habits and lifestyle: all these contribute in part to a positive energy balance, which leads to an increase in weight (Aronne et al., 2009). Any factor that leads to a reverse in the positive energy balance, turning it into a negative balance, will lead to a loss of weight (Fruhbeck et al., 2009).

Overcoming the problem of obesity requires a detailed understanding of the underlying causal mechanisms, followed by an implementation of the appropriate

intervention. Excess energy accumulates as additional adipose tissue. Reducing the amount of white adipose tissue (WAT) reduces the health consequences of obesity. Interestingly, brown adipose tissue (BAT) has recently received more attention due to its potential role in fighting obesity (Symonds et al., 2011; Carey and Kingwell, 2013; Vosselman et al., 2013).

### 1.1.4 Effect on Health

Overweight and obesity in infancy and puberty are closely connected to each other, both groups experiencing the augmented hazard of premature morbidity and mortality (Reilly and Kelly, 2010). Many diseases are related to obesity, including type 2 diabetes, high blood pressure, coronary thrombosis, heart failure, bronchial asthma, back ache, gallbladder diseases, and the augmented hazards of infirmity which, in total, cause more than three million deaths per year in the world (Guh et al., 2009; Ng et al. 2014). Research on obesity demonstrates that it is a major public health problem, decreasing life expectancy – especially in young people (Finucane et al., 2011). Unfortunately, these health consequences have a considerable effect on healthcare expenditures (Guh et al., 2009). Overweight and obesity are strongly correlated with chronic health complications, deterioration of health-related quality of life, increasing costs of medical care, and pharmaceutical expenses (Hayward and Colman, 2009).

## 1.2 The Adipose Organ

In all mammals, the adipose organ consists of at least two kinds of adipocytes, white and brown, located in depots in different areas of the body. The amounts of these adipocytes vary due to several factors. White and brown adipocytes differ in morphology, because they both contain variable amounts of triglycerides (TGs) and lipids. The colour of one depot, or part of it, is determined according to the colour of the adipocytes in it. If most of the adipocytes are brown, it is brown adipose tissue; if most of the adipocytes are white, it is white tissue (Cinti, 2006).

### 1.2.1 White Adipose Tissue

White adipose tissue (WAT) is the main depot where excess energy is stored, and is distributed in visceral and subcutaneous deposits (Wajchenberg, 2000). Visceral depots are sited near internal organs, such as the retroperitoneal, intra-abdominal and perigonadal regions. Subcutaneous depots are under the skin in the abdominal, inguinal and gluteal regions (Gesta et al., 2007). These positions vary between individuals and genders (Lemieux et al., 1993). WAT has two basic functions, the first is storage of energy in the form of TG in the case of positive energy balance, that is then released as required. The second function is to secrete a range of adipokines, including adiponectin and leptin, which plays an important role in regulating energy balance (Cinti, 2006). WAT is also important in glucose homeostasis (Shimomura et al., 1998; Moitra, et al., 1998) and the inflammatory process (Cousin, et al., 1999).

WAT represents the largest endocrine tissue in humans. The pleiotropic nature of WAT is based on the ability of fat cells to secrete numerous hormones, cytokines, enzymes, matrix proteins, growth factors and complement factors (Coelho et al., 2013). Due to these factors, WAT receptors regulate numerous processes, including metabolism homeostasis, energy expenditure, food intake, blood pressure homeostasis, and immunity (Ahima, 2005; Matsuzawa, 2006). The key role of WAT is to store triglycerides during fatty acid (FA) release and energy consumption when energy expenditure exceeds energy intake. Although WAT has been considered an inactive metabolic tissue, it regulates energy metabolism. This regulation is triggered by endocrine, autocrine and paracrine signals that permit the adipocyte to regulate the metabolism (Vazquez-Vela et al., 2008). The endocrine activity of WAT was assumed due to its ability for steroid hormone interconversion (Coelho et al., 2013). The discovery of Leptin in 1994 increased the list of adipocyte-derived factors which informed the field of adipocytes biology, endocrine and metabolic functions, and the functional associations between adipocytes secretions and peripheral metabolic functions (Fonseca-Alaniz et al., 2007; Saely et al., 2012).

Histologically, WAT is spherical, varying in size (depending on the size of the unilocular lipid droplets inside them). These droplets occupy most of the cytoplasm and squeeze the nucleus. However, the cytoplasm that surrounds the nucleus is thicker than in other areas in the cell (Figure 1.2). Under a microscope, mitochondria appear elongated, and other organelles can be seen. The cell is surrounded by an external lamina (Cinti, 2006).

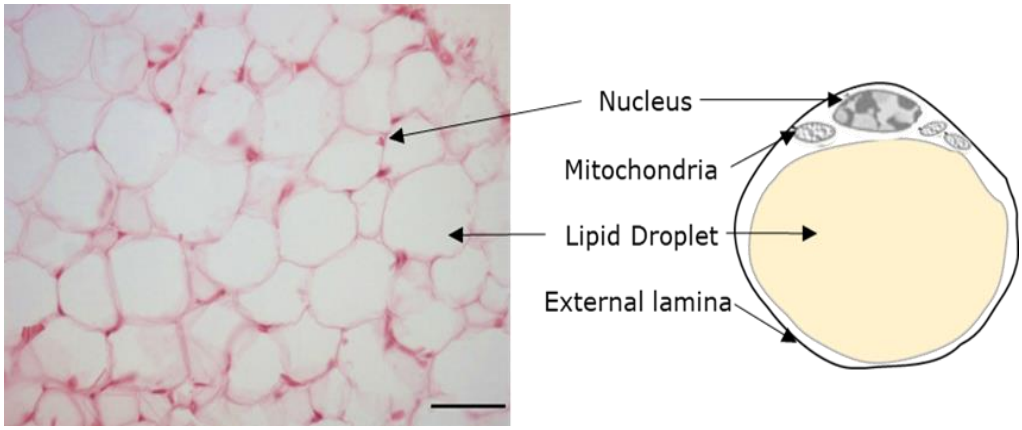



**Figure 1.2 Histological image and schematic diagram of white adipocyte.**
H&E stained microscopic histological section from perirenal depot in sheep at age of 28 days. White adipocytes are generally spherical and vary in size with a singular lipid droplet (unilocular) surrounded by cytoplasm containing the nucleus and a small number of mitochondria and external lamina surround the cell. Scale bar = 300 µml; magnification 20x.

### 1.2.2 Brown Adipose Tissue

Brown adipose tissue (BAT) is a recently rediscovered organ in adult humans. Although first described in the mid-sixteenth century, its presence in all mammals was confirmed only in the early twentieth century. It was originally considered that BAT was a functional organ only in neonates (Nedergaard et al., 2007), until recent studies demonstrated its operative role in adults (Enerback, 2010; Hairil Rashmizal et al., 2010; Nedergaard et al., 2011; Nedergaard et al., 2007; Virtanen et al., 2009).

BAT's function is characterised by expending rather than storing energy. Heat production occurs by non-shivering thermogenesis, mediated by the specific BAT

uncoupling protein 1 (UCP1). During thermogenesis, FAs are quickly removed from the surface of lipid droplets. Because BAT is multilocular, huge quantities of free fatty acids (FFAs) can be used by mitocondria (Di Paola & Lorusso, 2006). The mitochondria oxidise FAs, making a proton gradient that will be lost by the induction of protonophore UCP1, an ionophore which allows protons to cross lipid bilayers, and is uniquely expressed in BAT mitochondria internal membranes. The result of burning FAs is heat production (Cinti, 2006).

In addition to differing from WAT in function, BAT differs morphologically and at the molecular level. The subsequent sections will address these differences in more detail.

#### 1.2.2.1 Anatomical Locations

The location and abundance of the major BAT depots differ between species (Figure 1.3). For example, the major depot in human beings is in the supraclavicular area (Cypess et al., 2009; Cypess et al., 2013; Saito et al., 2009; Virtanen et al., 2009). Most BAT in mice is in the intrascapular area (de Jong et al., 2015; Frontini, et al., 2010; Vitali et al., 2012), an area also found in newborn humans that gradually disappears with age, and is only found very rarely in adults (Enerback, 2010). Other depots in humans are distributed in the following areas: thyroid/tracheal, mediastinal, paracervical, parathoracical, supra and perirenal (Enerback, 2010; Vosselman et al., 2013). In sheep, the largest depot is the perirenal depot followed by sternal and clavicular areas (Symonds et al., 2012).

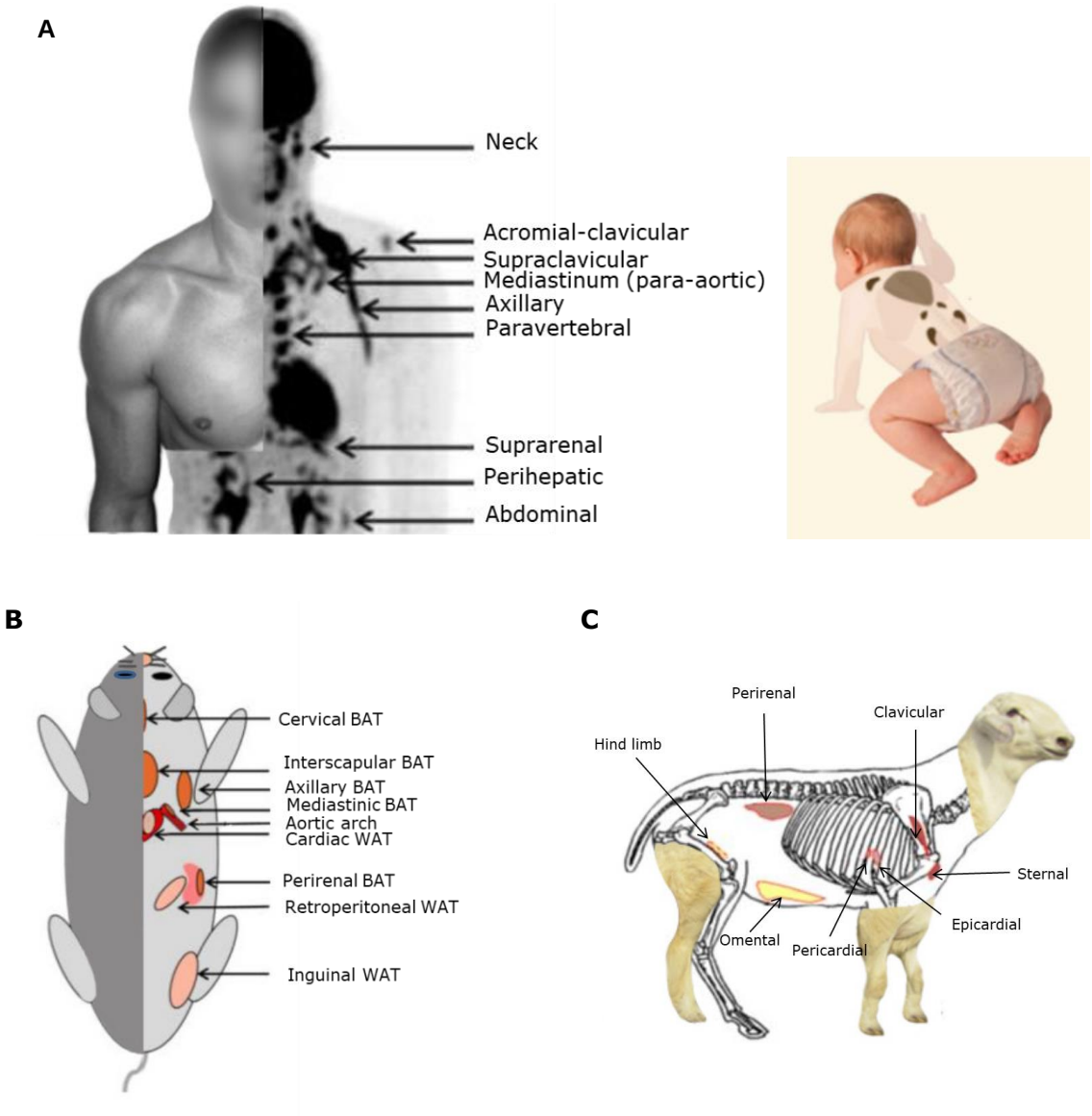



**Figure 1.3 Locations of BAT depots in (A) humans, (B) rodents and (C) sheep.**

The arrows indicate the BAT depots in the three species. The main depot in humans is in the supraclavicular area (third arrow on left), in rodents is the interscapular area (second arrow on right and in the perirenal area in sheep. In infants, BAT appear as a thin layer in the form of a kite. Diagrams adapted from Enerback (2010); Vosselman et al. (2013) and Symonds et al. (2012).

### 1.2.2.2 Morphological Composition

Histologically, the feature that distinguishes brown adipocytes from white is that they contain numerous, small, multilocular fat droplets, compared with a singular droplet in white adipocyte. Brown adipocytes have several large mitochondria packed with cristae and a round nucleus, which gives the tissue its brown colour (Cinti, 2006;

Fruhbeck et al., 2009) (Figure 1.4). Additionally, brown adipocytes are highly vascularised (Fruhbeck et al., 2009) but do possess similar organelles to WAT. Table 1.1 summarises differences in characteristics between BAT and WAT.

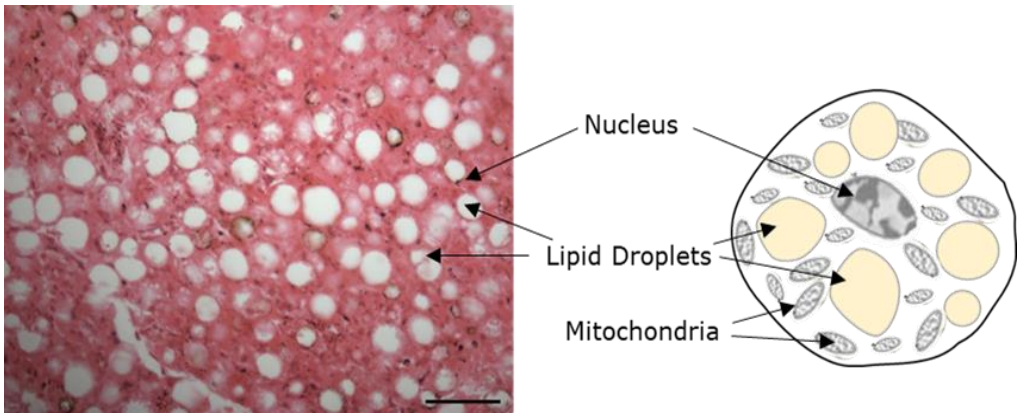



**Figure 1.4 Histological image and schematic diagram of brown adipocyte.**

H&E stained microscopic histological section from perirenal depot in sheep at age of 7 days. Brown adipocytes have multiple lipid droplets (multilocular) containing the nucleus and an abundance of mitochondria within the cytoplasm. Scale bar = 300 μm; magnification 20x.

**Table 1.1 Differences in characteristics between BAT and WAT. Adapted from Fruhbeck et al. (2009).**

| **Characteristic** | **WAT** | **BAT** |
|---|---|---|
| Function | Store energy<br>Release adipokine factors, such as leptin and adiponectin, which regulate energy balance | Thermogenesis |
| Main depot location | Visceral depots: retroperitoneal, intra-abdominal and perigonadal regions<br>Subcutaneous depots: abdominal, inguinal and gluteal regions | Thyroid/tracheal<br>Mediastinal<br>Paracervical/supraclavicular<br>Parathoracical<br>Perirenal and Supra-renal |
| Colour | White, ranging from light ivory to strong yellowish | Brown, ranging from light pinkish to dark reddish |
| Vascularisation | Adequate amount of vascularisation | Abundant flow of blood and vessels number |
| The nervous system | Primarily sympathetic and parasympathetic | Sympathetic |
| Adipocyte shape | Varies from polyhedral to spherical | Polygonal |
| Size | Variable | Much smaller |
| Nucleus | Flattened,Peripheral semilunar, occupying 2 – 3% cell volume | Central, oval or round |
| Lipid droplets | Large, unilocular, single, lipid droplet c. 90% of the cell volume | Multilocular, many small lipid droplets |
| Mitochondria | Small, few, elongated | Large, abundant, round |
| Cytoplasm | Thin, stretched into a slim rim | Stretched around the cells |

#### 1.2.2.3 Transdifferentiation

It may be possible to turn brown adipocytes into white. When there is no adrenergic stimulation, BAT gradually loses its brown characteristics and turns into a tissue like WAT, including loss of the UCP1 gene, activation of the leptin gene, and loss of blood vessels and nerves (Cinti, 2006). This process of transdifferentiating appears to be reversible. In other words, white adipocytes may be turned into brown. However, this process is difficult due to the difference between white and brown adipose tissue in their progenitor lineage (Park et al., 2014). The transdifferentiation process is based on the fact that, in rodents at least, the relative distribution of WAT and BAT differs according to environmental conditions, nutritional situation, species, age, and gender (Hu et al., 2010, Kwok et al., 2016). Thus, a change in one or more of these conditions may be associated with changes at the histological level in the form of increased BAT in white depots. For example, several studies have demonstrated that adipocytes that are white at a thermoneutral room temperature turn to brown at lower temperature (Enerback, 2010; Murano, 2005). Other studies have shown that the same change can be effected with pharmacologic treatments, such as when given to mice that are genetically obese and diabetic (Himms-Hagen et al., 2000; Jimenez et al., 2003).

#### 1.2.2.4 Non-shivering thermogenesis

Non-shivering thermogenesis (NST) is defined as 'a cold-induced increase in heat production that is not associated with the muscle activity of shivering' (Himms-Hagen, 1984). In humans and large mammals, UCP1 is activated in the mitochondria at birth when newborns are exposed to the cold extra-uterine environment (Cannon & Nedergaard, 2004) and is employed to produce heat by NST, thus countering loss of heat which would happen due to the high surface area-volume proportion (Kozak and Anunciado-Koza, 2008). UCP1 is an inner mitochondrial-membrane protein which characterises BAT, and uncouples respiration from oxidative phosphorylation. This uncoupling results in heat production, rather than utilising respiration to synthesise

adenosine triphosphate (ATP) (Cannon & Nedergaard, 2004). Further mitochondrial metabolite carriers are also connected with UCP1 to allow the transportation of protons from the mitochondrial intermembrane space to the mitochondrial matrix (Rial et al., 2010). Proton transportation is activated by FAs and repressed by purine nucleotides (Hoang et al., 2013).

BAT activation in animals is caused by an increase in sympathetic nerve activity, promoting glucose and lipid uptake (Cannon & Nedergaard, 2004). In humans, the uptake of glucose was first visualised in 1996 in the neck region of patients using positron emission tomography (PET) scans and 18 F-fludeoxyglucose (FDG) (Barrington & Maisey, 1996). By 2007, this evidence were used to identify the areas of glucose uptake as BAT (Nedergaard et al., 2007). Subsequently, many studies have been conducted on healthy adults (Saito et al., 2009; van Marken Lichtenbeltet al., 2009; Virtanen et al., 2009) and patients (Au-Yong et al., 2009; Cypess et al., 2009) to identify BAT depots. In general, an increase in glucose uptake – BAT activity – was observed in cold environments (Au-Yong et al., 2009; Cypess et al., 2009). It is worth mentioning that FA uptake (which is the primary substrate in BAT) was not measured in the scans (Bartelt et al., 2011) when radiolabelled glucose was used.

#### 1.2.2.5 BAT Activation

Activation of UCP1 is caused by a signalling cascade that starts with norepinephrine (NE) (or noradrenaline). NE is released by sympathetic nervous system terminals onto the β3 adrenergic receptor on the membrane of plasma (McCorry, 2007). This catalyses adenylyl cyclase (AC), an enzyme with key regulatory roles in most cells, which activates the transformation of ATP to cyclic adenosine monophosphate (cAMP). Following this, cAMP catalyses cyclic AMP-dependent protein kinase (PKA) which frees its active catalytic subunits (C) from its regulatory subunits (R). Thus, PKA phosphorylates hormone-sensitive lipase (HSL) resulting in the stimulation of lipolysis (Ahmadian et al., 2007). Lipolysis hydrolyses triglycerides into glycerol and FFAs, while long-chain fatty acids (LCFAs) then bind to UCP1, activating it (Figure 1.5).

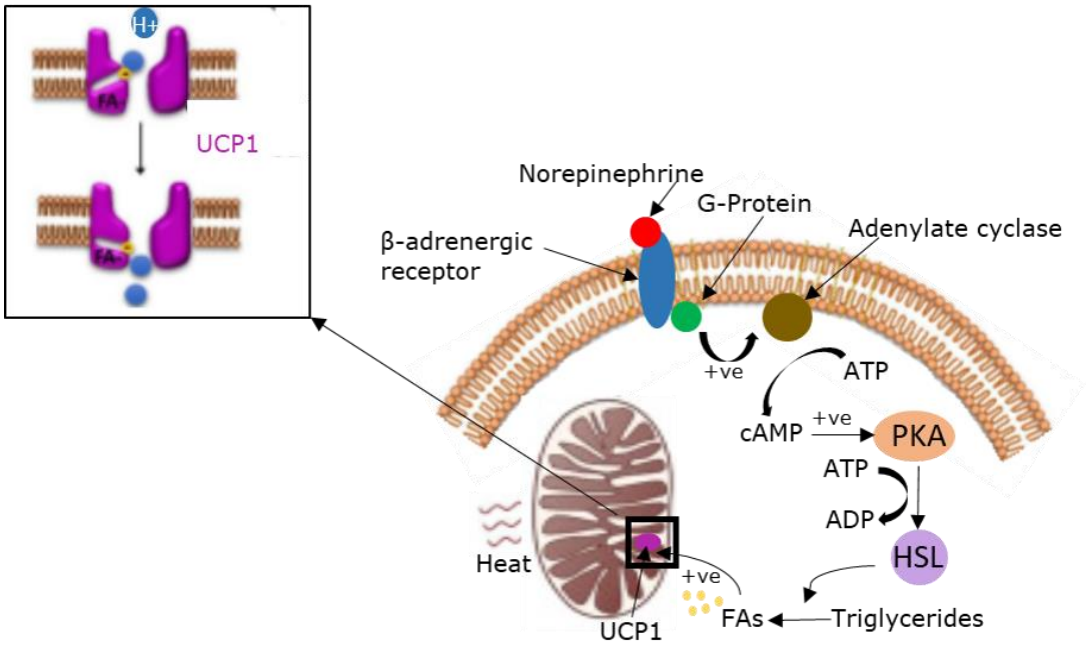



**Figure 1.5 UCP1 activation by β-3 adrenergic cascade.**

In response to cold, norepinephrine binds to the β-3 adrenoceptor which result in activating adenylate cyclase which, in turn, generates cyclic adenosine monophosphate (cAMP). This activates protein kinase A (PKA) directing the reaction to phosphorylate and activate hormone sensitive lipase (HSL) which when activated accelerates fatty acid (FAs) release from stored triglycerides. Some FAs activate the uncoupling protein (UCP) 1 that uncouples the proton gradient in the inner membrane of mitochondrial, thus, releasing the energy in form of heat. Diagram adapted from Divakaruni & Brand (2011).

Many genes which are affected by FAs supplementation have important roles in fat metabolism: these genes may affect UCP1 activation. The *ACOT11* gene encodes for acyl-coenzyme A thioesterase 11, a β-oxidation pathway enzyme that plays a role in lipid anabolism by antagonising lipid catabolism (Ellis et al., 2015). Enzymes of the acyl-coenzyme A thioesterase family convert fatty acyl-coenzyme A to FFAs and coenzyme A molecules, regulating FFAs metabolism by controlling substrate availability during oxidation, and regulating the intracellular trafficking of fatty acyl-coenzyme A (Kathayat et al., 2018). The absence of *ACOT11* expression results in an increased rate of FA oxidation (Zhang et al., 2012).

The expression of *UCP1* could be influenced by FAs synthesis. For example, the expression of *FADS2* encodes the enzyme fatty acid desaturase 2, which catalyses the desaturation of LCFAs to PUFAs (Glaser et al., 2010), has been reported to decline in liver and adipose tissue after supplementing diet with PUFAs (Raclot & Oudart, 1999; Xu et al., 1999). Essentially, this means reducing the LCFAs available to bind to *UCP1* activation (Sul et al., 1998). *FABP3* encodes fatty acid binding in protein 3, which is a carrier protein involved in the intracellular transportation of LCFAs for storage (York, 2013). Its levels are a determinant of FA oxidation efficiency by brown adipose tissue, representing a potential target for the modulation of energy dissipation (Vergnes et al., 2011).

A gene that has been associated with the suppression of UCP1 is receptorinteracting protein (*RIP*) 140 (Debevec et al., 2007). It has been suggested that it is suppresses the browning of WAT because *RIP140* knockout mice are lean, and have increased *UCP1* expression in WAT (Leonardsson et al., 2004).

#### 1.2.2.6 Factors affecting BAT activity

Histological studies, whether in large mammals or humans, proved the presence of BAT in new-borns, which then quickly disappears (Heaton, 1972). However, as reviewed above, the progress in clinical studies and the development of new imaging modalities recently proved the presence and metabolic activity of BAT in adults. Since this discovery, many studies have explored how to activate BAT in humans.

##### 1.2.2.6.1 Ambient Temperature and Season

Most studies have shown that BAT can be triggered by exposure to cold temperatures in the range of 17±1°C (Lichtenbelt et al., 2009; Saito, 2013; Vosselman et al., 2013); no activity was noticed at higher temperatures. One possible explanation is that exposure to cold increases norepinephrine (NE) levels in plasma, which activates the sympathetic nervous system (SNS) (Orava et al., 2011; Vosselman et al., 2012).

Seasons affect BAT activity in two ways. First, BAT activity is increased more in winter than in summer because of low temperatures: in a study of 56 healthy people between the ages of 23 - 65, it was found that BAT activity increased in winter (Au-Yong et al., 2009, Saito et al., 2009). Secondly, the effects of photoperiod compared with ambient temperature is less uncertain. For example, Siberian hamsters exposed to short winter days for five weeks exhibited enhanced BAT function with a marked increase in fat storage (Demas et al., 2002). This may be the result of prolactin release, which is affected by both increasing the length of the day and decreasing the temperature (Goldman et al., 1981). It has been proven that the length of the day is the main determinant of the prolactin concentration in sheep mothers' plasma (Kennaway et al., 1987) which is inversely correlated with the abundance of prolactin receptors (PRLR) (Auchtung et al., 2005). PRLR are represented in foetal adipose tissue (Symonds et al., 1998) and considered a potential mediator that could change adipose tissue composition through maternal nutrition (Symonds et al., 2011).

#### 1.2.2.6.2 Age

BAT activity declines with age. For example, Yoneshiro et al. (2011, 2013), in a study of 213 healthy people aged between 20 and 73 years, found that the cold activated BAT by more than 50% among those in their twenties, but less than 10% among those in their fifties and sixties. In children, the prevalence was greater than 40% under conventional heat conditions, compared with less than 10% among adults under the same conditions. The highest activity was in the 13 to15-year group (Drubach et al., 2011; Gilsanz at al., 2012). Results from thermal imaging studies demonstrate the decrease of thermal activity with the increase of age in the supraclavicular region (Symonds et al., 2012).

#### 1.2.2.6.3 BMI and Adiposity

BAT activity and BMI are inversely related in children and adults (Robinson et al., 2014, Saito, 2013). It has been found that cold-activated BAT is also reduced in the healthy obese (van Marken Lichtenbelt et al., 2009; Saito et al., 2009; Yoneshiro et

al., 2011). For example, van Marken Lichtenbelt et al. (2009), in their study on 24 healthy people aged between 18 and 32, had 10 with a normal BMI (<25) and 14 that were overweight and obese (BMI ≥25). They found that BAT activity was lower as BMI was raised.

#### 1.2.2.6.4 Food Ingredients

It has been demonstrated that cold exposure is the most effective physiological condition to activate BAT in humans (Saito et al., 2009) through beta-adrenergic receptors (Sakamotoa et al., 2014) and via transient receptor potential channels (TRPs) (Caterina, 2001). However, the effects of cold exposure could be simulated through chemical activation of TRPs. Some food ingredients have been identified as TRP stimulators, such as menthol (a compound of mint-flavour ingredients) (Ma et al., 2012) and capsaicin, which gives the chili pepper a pungent taste (Ludy et al., 2012 Snitker et al., 2009; Whiting et al., 2012). Sakamotoa et al. (2014) suggest that the continuous stimulation of the TRPs by food compounds could be effective ways to maintain BAT.

### 1.2.2.7 BAT and Obesity

Many people who suffer from obesity do not have problems losing weight: rather, the most common problem is a lack of continuity in maintaining a new weight (Carey & Kingwell, 2013). There are many factors behind this, including a reduction in basal metabolic rate (BMR) (Sumithran et al., 2011). Leibel et al. (1995) found that the BMR was 20% less in obese people who lost part of their weight compared with others who did not suffer from obesity, but had the same weight.

There are two conclusions to be made: first, any action that leads to a negative energy balance – more expended calories than intake – can have an impact on weight loss. The second is that one role of BAT is to dissipate energy directly as heat. It is possible that the activation of BAT contributes to weight loss, while maintaining this loss by increasing energy expenditures occurs only when other factors, such as food

intake, remain stable (Christians & Garby, 2002). Many studies indicate an inverse association between obesity and BAT activity (Vosselman et al., 2013).

The first proposal for BAT as an aid to fight obesity was developed more than 35 years ago (Himms-Hagen, 1979), followed by recent studies that demonstrate the usefulness of BAT activation in obese persons through exposure to cold or insulin, or due to sympathomimetic administration (Carey & Kingwell, 2013). In adults, full activation of BAT depots enable an energy burn equivalent to 4 kg of WAT over a one-year course (Carey & Kingwell, 2013). In rodents and humans, 40-50 g of BAT could contribute up to 20% of the total daily energy expended when fully stimulated (Rothwell & Stock, 1979; 1983) and 5% of BMR (van Marken Lichtenbelt, & Schrauwen, 2011). Consequently, it is conceivable that a lack of BAT activation may contribute to the development of obesity.

#### 1.2.2.8 Major BAT depots

BAT depots are in several locations throughout the body, which vary between species. In humans, the main BAT depot is in the supraclavicular and neck area, while in rodents, the interascapular depot is the largest (Nedergaard & Bengtsson, 2007). Other depots in humans include the thyroid/tracheal, mediastinal, parathoracical, supra and perirenal (Enerbäck, 2010). In sheep, perirenal, sternal and epicardial are major BAT depots, known to be populated with brown adipocytes early in postnatal life (Symonds et al., 2012).

##### 1.2.2.8.1 Perirenal Adipose Tissue

Perirenal adipose tissue (PAT) is the depot for fat that surrounds the kidney. It is considered a main depot for fat in the sheep foetus, comprising up to 80% of its total fat. The volume and weight of PAT increases in late gestation. For example, Alexander (1978) found an increase in the amount of PAT in foetal Merino sheep by 34% during the last three weeks of gestation. In a similar fashion, Vernon et al. (1981) reported a 40% increase in the adipocyte volume of foetal lambs in the last four weeks of

gestation, and the majority was in PAT mass: adipocyte hypertrophy. In newborn calves, the increase in PAT mass starts from the last trimester, about 96 days before birth, in conjunction with an increase in the UCP1 concentration (Smith et al., 2004). However, 48 days from birth, no increase was observed in PAT mass or adipocytes volume.

Morphologically, PAT adipocytes contain many large mitochondria with well-differentiated cristae. As a pregnancy approaches term, morphological changes occur in fetal PAT adipocytes. Smith et al. (2004) demonstrated the changes in PAT mitochondria from large and spherical at the beginning of the third trimester to elongated by parturition, and the cristae became highly convoluted and differentiated when earlier in gestation, they were poorly defined.

#### 1.2.2.8.2 Epicardial Adipose Tissue

Epicardial adipose tissue (EAT) is a visceral thoracic fat consisting of a mesothelial cell population that migrates from the area of the septum transverse to the surface of the heart (Sacks & Fain, 2007). In human adults, EAT is normally found in the interventricular and atrioventricular grooves (Willliams, 1995). Surrounding 80% of the heart's surface,  it also represents approximately 20% of a normal heart's weight (Shirani et al., 1995) – with some variation between men and women (15.2%–25.2% and 19.5%–21.7%, respectively) (Rabkin, 2007; Shirani et al., 1995), as well as between individuals of the same sex (4%–52%) (Roberts & Roberts, 1983; Shirani et al., 1995). However, epicardial fat may weigh more than recorded to date, as it is not possibile to completely autopsy the myocardium (Corradi et al., 2004; Shirani et al., 1995).

EAT has been described as a BAT, since it is dynamically involved in energy metabolism and lipids (Iacobellis et al., 2005; Rabkin, 2007; Sacks & Fain, 2007). EAT properties have been distinguished from other fat depots in several ways, including but not limited to high rates of release and uptake FFAs, low rates of glucose utilisation, and slow regression during weight loss (Iacobellis & Bianco, 2011; Rabkin,

2007; Sacks & Fain, 2007). Hence, it can be considered as a structural barrier, saving the heart from high exposure to FFAs, and to provide energy for the myocardium (Iacobellis et al., 2005). Conversely, increased circulating FFAs result in more storage within EAT: this could be a potential risk factor for cardiac diseases (Iacobellis et al., 2005).

Morphologically, epicardial adipocytes are usually smaller than those in other fat deposits, specifically omentum and pararenal deposits (Sons & Hoffmann, 1986): this could be due to low amounts of mature adipocytes compared to preadipocytes (Iacobellis & Bianco, 2011). The epicardial adipocyte cell size in sheep was the smallest among seven other deposits, while the per gram number of adipocytes tissue was the greatest in EAT (Barber et al., 2000).

The prevalence of obesity has attracted attention to visceral abdominal adipose tissue, including EAT, as a risk factor for obesity and related diseases (Sacks & Fain, 2007). Since the total weight of EAT significantly correlates with body weight, obesity results from an increase in its amount (Lacobellis et al., 2004; Sons & Hoffmann, 1986). Regarding the relationship between the amount of EAT and BMI, the results of autopsy studies vary between finding no correlation (Shirani et al., 1995) or a significant weak correlation (Corradi et al., 2004). In terms of visceral abdominal adiposity, although there is little clinical research, data strongly supports the relationship with EAT (Rabkin, 2007). It is well known that individuals with high abdominal visceral fat are at high risk of having or developing atherosclerotic cardiovascular disease (Isomaa et al., 2001).

#### 1.2.2.8.2 Sternal Adipose Tissue

Sternal adipose tissue in large mammals is a comparable depot to the supraclavicular depot, the main BAT depot in adult humans (Cypess et al., 2009; Virtanen et al., 2009). It is located where the sternal and clavicular areas meet the forelimb (Symonds et al., 2012). Located at the front of the animal, immediately above the skeletal muscle where foetal blood supply is sustained, it is likely important in foetal

development. Additionally, the BAT deposit could be essential for maintaining brain temperature after birth due to its nearness to major vessels supplying the brain (Symonds et al., 2012). In sheep, Henry et al. (2017) demonstrated that sternal adipose tissue was one of the primary sites of thermogenesis alongside retroperitoneal and skeletal muscle. They showed that sternal fat development shares some characteristics with skeletal muscle, suggesting a correlation with the retention of UCP1 in adulthood. The related UCP1 gene expression was greater than double out of all other adipose tissue depots in three months' - old sheep (Symonds, 2012). Similarly, the abundance of UCP1 was higher in sternal fat compared with retroperitoneal fat, and was retained in adulthood (Henry et al., 2017): additionally, UCP1 demonstrated a greater thermogenic response to the diet. Therefore, sternal adipose tissue could be a potential target to dietary interventions aimed to promote thermogenesis in large mammals.

### 1.2.3 Brite Adipose Tissue

In addition to brown and white adipocytes, there is another type of adipocytes called brite (brown-in-white) or beige (Klingenspor, 2012) which was first discovered in rodents (Young et al., 1984). Brite/beige fat is identified as inducible BAT dissipating energy at cold temperatures (Cousin et al., 1992; Fukui et al., 2000; Guerra et al., 1998) and working as an extra reserve of BAT (Hoffmann et al., 2015). Brite adipocytes are scattered among the white adipocytes and are similar in form and structure to a classical brown adipocyte (Figure 1.6) comprising multilocular cells, but subject to greater size variation, and are UCP1-positive mitochondria and contain lipid droplets (Bostrom et al., 2012). However, their lineage is different from classical brown adipocytes, as will be discussed further (Section 1.2.4). Although brite adipocytes express some specific BAT genes, including UCP1, they have a distinct pattern of gene expression, and have their own genetic markers, such as homeobox (HOX) C9 and short stature homeobox (SHOX) 2 (Walden et al., 2012).

There is no accord regarding the mechanism of "browning" and the embryonic derivation of brite adipocytes. Some studies argue that brite adipocytes appear because of previously existing white adipocytes (Vitali et al., 2012). In contrast, another study argues that they emerge by *de novo* adipogenesis from different precursors (Wang et al., 2014). Another study proved that in inguinal (igWAT), 10 % of brite adipocytes arise from smooth muscle (Long et al., 2014). It seems that brite adipocytes may be more heterogeneous when compared to other adipocytes (Sanchez-Gurmaches & Guertin, 2014).

Having a similar thermogenic capacity to brown adipocytes (Okamatsu-Ogura et al., 2013; Shabalina et al., 2013) brite fat was investigated as a potential target to treat and prevent obesity, given its ability to increase energy expenditure.

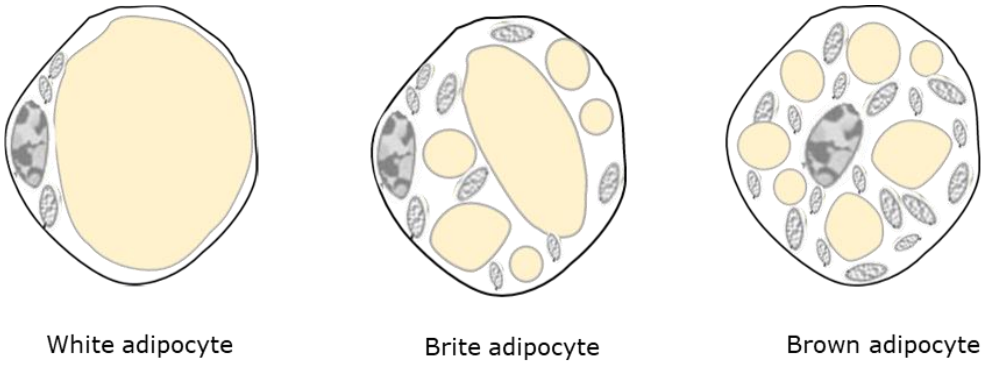



**Figure 1.6 White, brite and brown adipocytes.**
Brite adipocytes (in the middle) are dispersed among the white adipocytes and are morphologically similar to a classical brown adipocyte. They contain multilocular lipid droplets which vary in size and plenty of UCP1-positive mitochondria.

### 1.2.4 Development of adipocytes

The three different types of adipocytes (BAT, WAT and brite) are derived from mesenchymal stem cells (MSCs); however, the exact mechanism of their development is not completely known (Guilak et al., 2006). It was believed that all adipocytes had

the same progenitors until the detection that BAT and skeletal muscle (excluding WAT) share a common precurose in the form of myogenic factor (MYF) 5 progenitors (Myf5+) mediated by the action of PR domain containing (PRDM)16 (Seale et al. 2008). WAT adipocytes developed from (MYF5-) progenitors similar to those of brite adipocytes (Seale et al. 2008) (Figure 1.7). However, other studies have shown that brite adipocytes could emerge from a smooth muscle-like origin (Long et al., 2014). One study suggested that BAT, WAT and possibly brite adipose tissues were heterogeneous, having adipocyte progenitor cells from both MYF5+ and MYF5− lineages (Sanchez-Gurmaches et al., 2012).

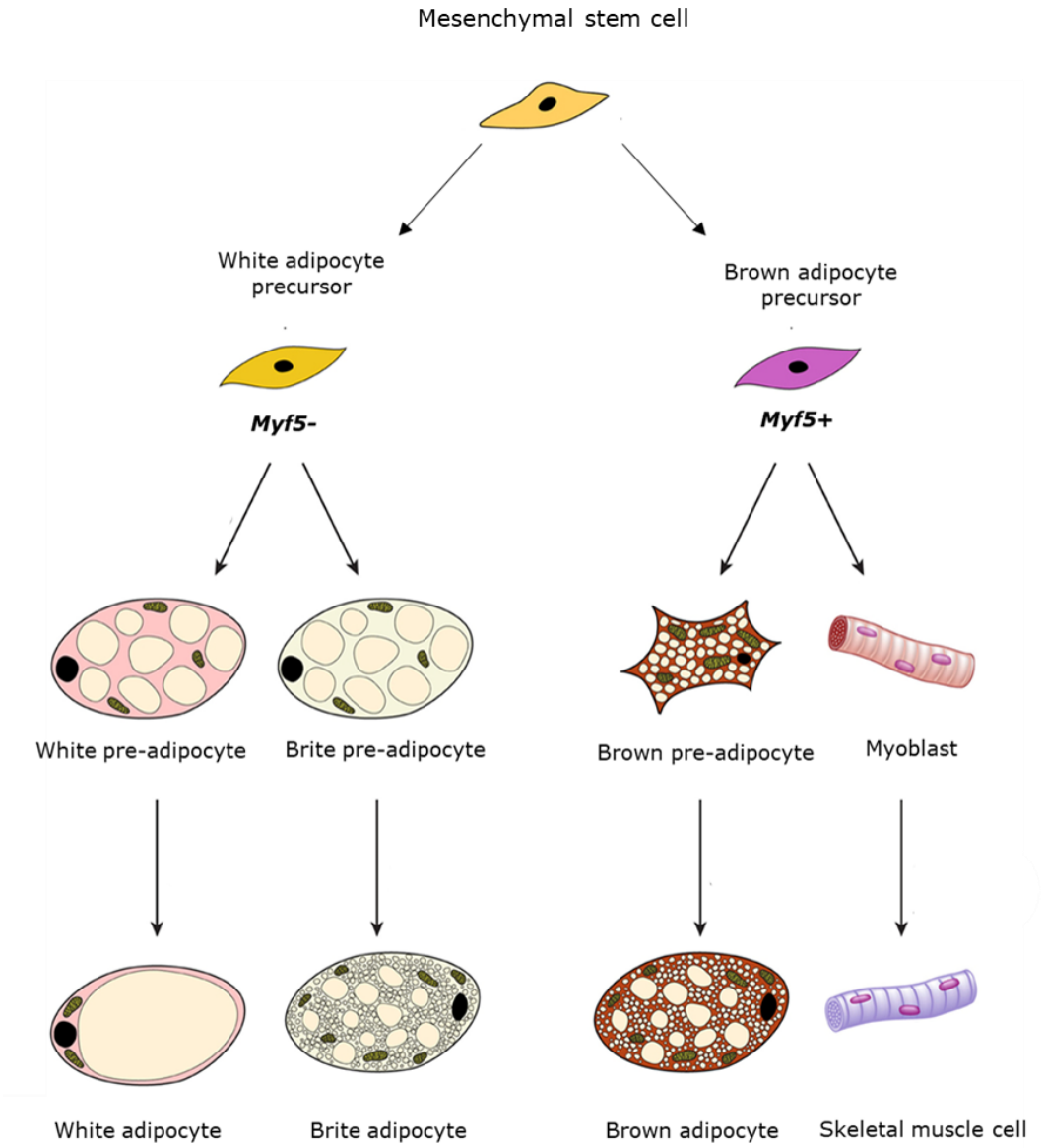



**Figure 1.7 The developmental pathways of adipocytes.**

White, brite and brown adipocytes all arise from pluripotent mesenchymal stem cells. Brown adipocytes are derived from myogenic factor (MYF) 5+ precursors, whilst white and brite adipocytes are derived from MYF5- precursor cells. The exact lineage of adipocytes is not clearly understood. Diagram adapted from Reddy et al. (2014).

## 1.3 Fatty Acids

Fatty acid (FA) is a carboxylic acid with a long aliphatic chain. These acids mostly occur naturally, and have an unbranched chain (either saturated or unsaturated) of evenly numbered carbon atoms (from 4 to 28) (Nic et al., 1997). Generally, these acids are obtained from phospholipids or triglycerides. Like glucose, these FA are vital nutritional sources of fuel due to their ability to produce ATP in large quantities after metabolism. However, the long chain fatty acids (LCFAs) with 14 or more carbons cannot pass the blood-brain barrier (BBB) and, therefore, cannot be used by the central nervous system cells as fuel. In contrast, medium-chain fatty acids (MCFAs) with 6 – 12 carbons and short-chain fatty acids (SCFAs) with fewer than 6 carbons can pass the BBB alongside glucose and ketone bodies (Vijay and Morris, 2007).

### 1.3.1 Fatty Acids Nomenclature

FAs are hydrocarbon chains found in triglycerides and phospholipids. The names of FAs are created according to the number of carbons, saturation level, and the position and geometrical configuration of the double bonds (if any are present). When FAs have no double bonds, they are saturated fatty acids (SFAs), are linear in structure, and solid at room temperature. If FAs have double bonds, they are unsaturated (USFAs): either monounsaturated (MUFAs) with one double bond, or polyunsaturated (PUFAs) with two double bonds or more (Lobb & Chow, 2007). USFAs usually have a bent structure, are liquid at room temperature and may occur in a two structural disposition of cis and *trans* isomers. Cis and *trans* FAs differ regarding the location of the hydrogen atoms attached to the carbon double bond; when the atoms are on the same side, the FA is cis, and when they are on different sides, the FA is trans (Figure 1.8). Typically, *trans* FA's do not exist naturally, but are produced industrially by hydrogenation (Katan and Zock, 1995).

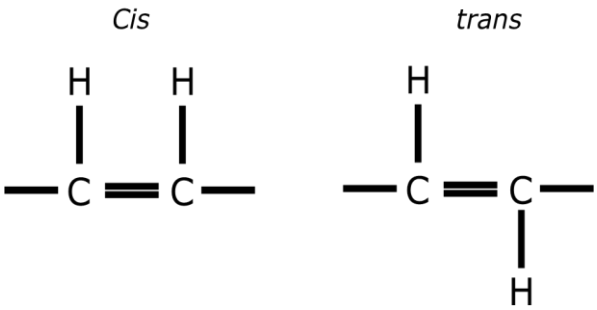



**Figure 1.8 Cis and trans configurations of carbon double bonds in fatty acids.**

Cis indicates that the functional groups are on the same side of the carbon chain while *trans* conveys them on opposing sides.

The carbon atoms of UFAs can be numbered by two systems: the Δ numbering system, that numbers the atoms from the carboxyl group; or the n/ω numbering system, which begins numbering at the furthest carbon atom from the carboxyl group (Katan and Zock, 1995; Nic et al., 1997). In the Δ numbering system, the number of carbon atoms is listed, followed by the number of double bonds, then the sign Δ, followed by the position of the double bond relative to carboxyl group end. For example, 16:1 Δ9 refers to the FA that has 16 carbon atoms and one double bond, located nine carbon atoms away from the carboxyl group, which is palmitoleic acid. In the n/ω numbering system, palmitoleic acid would be itemised as 16:1 (n-7), where number 7 refers to the position of the double bond in the 7th carbon atom away from the ω-carbon atom (the furthest carbon atom from the carboxyl group).

### 1.3.2 Essential Fatty Acids

The reason for naming these FAs as 'essential' is that they are extremely important to maintain bodily growth, development, and function. Essential fatty acids (EFAs) are characterised by their inability to be synthesised from simple carbon precursors in mammalian bodies, due to the lack of certain desaturases (Christie, 1982; Le et al., 2009): therefore, they must be obtained from dietary sources. Fundamentally, there are two EFAs identified as α-linolenic acid (ALA, omega-3) and linoleic acid (LA,

omega-6) which are essential to synthesising all downstream FA's (Das, 2006; Le et al., 2009; Voet, 2004). LA and ALA cannot be synthesised by mammals due to the lack of Δ12 and Δ15 desaturases that prevent double bonds beyond the ninth carbon atom in the chain (Christie, 1982). The richest dietary sources of LA are seed oils such as safflower, sunflower and soya bean (AbuGhazaleh et al., 2007). ALA sources include oils of linseed, rapeseed, soya bean, oats, with low quantities found in sunflower oil (Shingfield et al., 2010).

On the other hand, some FAs could be considered essential, only under certain conditions. For example, arachidonic acid (AA, omega-6) can replace LA as the only source of dietary omega-6 FAs in case of EFAs deficiency (when EFAs provides less than 1-2% of total calories) (Hansen & Jensen, 1985; Hansen et al., 1986; Thomasson, 1962; Wene et al., 1975). In similar fashion, docosahexaenoic acid (DHA, omega-3) and eicosapentaenoic acid (EPA, omega-3) could be more effective and efficient than ALA (Bjerve et al., 1987; de Groot et al., 2004; Lauritzen et al., 2001; van Houwelingen et al., 1995; Williams & Burdge, 2006).

### 1.3.3 *Trans* Fatty Acids

*Trans* fatty acids (TFAs) are UFAs that have a minimum of one carbon-carbon double bond with hydrogens in the trans-configuration. They arise either by biohydrogenation (BH) in animal rumen, or by partial hydrogenation of unsaturated oils (Wanders et al., 2010). Ruminant TFAs which arise from biohydrogenation are present in small amounts in animals milk and meat, and include conjugated linoleic acid (CLA) and vaccenic acid (Ghafoorunissa, 2008; Iqbal, 2014). It is difficult to determine TFAs consumption in various countries due to their varied content in the diet. Nevertheless, it has been estimated to contribute 2-3% of the total energy intake in the USA, and 7% in South Asian and Middle Eastern countries (Sundram et al., 2003). Despite several epidemiological studies that found a relationship between industrial TFAs and cardiovascular disease (CVD), there is no evidence of such a relation with ruminant TFAs

(Ascherio et al., 1999; Bolton-Smith et al., 1996; Lock et al., 2005; Van de Vijver et al., 2000; Weggemans et al., 2004).

### 1.3.4 Fatty Acid Biohydrogenation in the Rumen

Milk fats are synthesised in the mammary epithelial cells from FFAs. About half of these FFAs can be obtained from lipids in the bloodstream (short and medium-chain fatty acids); the other half, from *de novo* synthesis in the mammary epithelial cells, (usually LCFAs) (Bauman & Griinari, 2003; Harvatine et al., 2009; Jensen, 1995; Lopez-Lopez et al., 2002; Park, 2009).

### 1.3.5 Mechanism of FAs Metabolism in BAT

FAs synthesis, metabolism, and storage are vital in thermogenesis because these processes are essential for the activity of UCP1 (Divakaruni et al., 2012; Fedorenko et al., 2012). Primarily, there are two mechanisms to get lipids in BAT: FA uptake via lipoprotein carriers and lipogenesis; or, *de novo* FA synthesis (Calderon-Dominguez et al., 2016). The main source of FAs in BAT are the enzyme lipoprotein lipase (LPL) that binds at the endothelial cell surface during the FAs synthesis in brown adipocytes (Bartelt et al., 2012). Chylomicrons and very low-density lipoproteins (VLDL) transport lipids obtained from the diet via lymphatic vessels into the bloodstream (Calderon-Dominguez et al., 2016). As soon as triglyceride (TG) rich-lipoproteins arrive in the blood, LPL hydrolyses them into FFAs and monoacylglycerol (MG) for BAT uptake. Bartelt et al. (2011) have shown that FAs uptake increases in mice under cold exposure, and is higher in BAT when compared to skeletal muscle. The increase of LPL expression and activity, which was caused by the β3-adrenergic pathway, enhances BAT FA flow. In contrast, studies on mice found that an increase in LPL activity leads to adiposity and insulin resistance (Duivenvoorden et al., 2005) and the expression of lipogenic genes (Bartelt et al., 2013). Finally, FAs are taken up into cells by plasmatic membrane receptors, then transferred by FA binding proteins (FABP) for further use or storage (Calderon-Dominguez et al., 2016).

FAs can be synthesised by lipogenesis in the cytosol. Firstly, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Following this, several reactions are catalysed by FA synthetase (FAS). Finally, palmitic acid (16:0) is generated. In BAT, the last stage of lipogenesis is completed by elongating very long chain fatty acids (ELOVL) 3 and stearoyl-CoA desaturase (SCD)1. Synthesised FAs can be esterified before oxidation or storage as TG in lipid droplets (Calderon-Dominguez et al., 2016).

### 1.3.6 Effects of FAs on Body Composition

Body composition and weight regulation are both affected by the quantity and profile of the FAs consumed in the diet. For example, SFAs have been shown to increase weight gain rates and obesity when compared with other FAs in adult humans (Bell et al., 1997; Larson et al., 1996; Westerterp & Goran, 1997). Concerning PUFAs, types of FAs, as well as other factors, have different effects on body composition. n-3 PUFAs have a negative correlation with body fat in both humans (Larson et al., 1996) and rats (Fernandez et al., 1996; Hill et al., 1992; Ikemoto et al.,1996). Fish oil, a source for EPA and DHA omega 3's, is known to reduce the rate of weight gain more than other oils (Ikemoto et al., 1996; Somova et al., 1999) as well as having a tendency to reduce fat accumulation (Hill et al., 1993; Hun et al., 1999). However, n-6 PUFAs have adverse effects on body weight as many studies have reported: its weight gain induction (Hill et al., 1992, Ikemoto et al., 1996) increases either fat cell size (van Amelsvoort et al., 1988) or number (Cleary et al., 1999).

The differential effects of FAs on body weight could be explained, in part, by their rates of oxidation. In rats, feeding different types of FAs that varied in their saturation degree resulted in lower oxidation rates in the group fed by SFAs than those fed by PUFAs (Leyton et al., 1987). For PUFAs, FAs vary in their oxidation rates. Linolenic acid (LA, 18:2 n-3) has more effective oxidation rates than linoleic acid (LA, 18:2 n-6) (DeLany et al., 2000). FAs oxidation rates are inversely associated with fat storage (Pellizzon et al., 2002).

In addition to the differential effect on total body fat, FAs vary in their effects on fat mass. Pellizzon et al. (2002) reported an increase in fat mass in the subcutaneous depot in rats fed soybean oil (n-6 PUFA) compared with those that were fed fish oil (n-3 PUFA) or palm oil (SFAs). In contrast, n-3 LCPUFAs reduce abdominal fat compared with SFAs or n-6 PUFAs (Hill et al., 1993, Hun et al., 1999; Pellizzon et al., 2002).

#### 1.3.6.1 Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is a FA which presents a linoleic acid isomer (C18:2, n-6), which in turn, was found to has an effect in reducing body weight in different species such as cows and sheep (Churruca et al., 2009), rats (Botelho et al., 2005; Kloss et al., 2005), and humans (Blankson et al., 2000; Gaullier et al., 2005). Most human studies on CLA supplementation examine its capacity to alter body composition: an increase in lean mass and a reduction of fatty mass (Chen et al., 2012; Kamphuis et al., 2003; Santos-Zago et al., 2008; Whigham et al., 2007). The reduction in weight with CLA supplementation could be attributed to its influence on UCP1, which enhances the β-oxidation capacity (Peters et al., 2001). It is likely that CLA interacts with PPARγ, resulting in increased expression of UCP1: thus increasing the capacity for lipolysis and weight loss (Lehnen et al., 2015). On the other hand, the decrease in fat mass with CLA supplementation could be due to a decrease in adipocyte size rather than its number (Azain et al., 2000).

In contrast, other human studies have not found any effect of CLA supplementation on fat accumulation (Zambell et al., 2000) or BMI (Berven et al., 2000; Medina et al., 2000). These conflicting results can be explained, partly, due to variations in supplementation doses and duration, as well as the individual isomer ratios.

### 1.3.7 UCP1 Gene Expression and FAs

UCP1 expression is affected by the type of fat (the FA profile), and quantity consumed. Supplementing a mouse diet with PUFAs (4.3% vs 1.6%, in the context of a standard, 9% fat content) for 2-3 weeks was found to increase UCP1 protein

abundance by 41%, which suggests that the impact of a diet high in PUFAs enhances the thermogenic capacity of BAT (Sadurskis et al., 1995). The expression of UCP1 was found to be induced in adipose tissues of mice fed with fish oil (a source of n-3 EPA and DHA) (Kim et al., 2015). This is likely to be due to the influence of fish oil on the SNS. Fish oil increases the expression of β3 adrenergic receptors (Kim et al., 2015) which bind NA, causing an activation of UCP1 (see Section 1.2.2.5). On the other hand, another study supplemented mice with EPA and DHA, found no effect on UCP1 (Janovska et al., 2013). In vitro, treating human pre- and mature adipocytes caused UCP1 upregulation with EPA but not DHA (Fleckenstein-Elsen et al., 2016).

In addition to PUFAs, MUFAs have been reported to increase UCP1 in adult rats fed a high fat diet (Rodríguez et al., 2002). Similarly, maternal supplementation with olive oil (high in MUFAs) during pregnancy and lactation caused a reduction in mother's weight gain and increased UCP1 in offspring's BAT (Priego et al., 2013). In humans, using olive oil as a source of dietary fat stimulated diet-induced thermogenesis (DIT) in postmenopausal women (Soares et al., 2004) (DIT is defined as 'the increase in energy expenditure above basal fasting level divided by the energy content of the food ingested') (Westerterp, 2004). This means that part of consumed calories will be expended as energy (Cannon & Nedergaard, 2012).

## 1.4 Milk

### 1.4.1 Composition of Milk Fat

Generally, milk fat contains 60-70% SFAs (e.g. myristic and palmitic acids) (Yu et al., 1995; Dewhurst and Lee, 2004), 25-35% MUFAs (e.g. oleic acid) and up to 5% PUFAs (e.g. α-linoleic, linoleic and conjugated linoleic acids) (Jensen, 2002; Corl et al., 2003; Dewhurst and Lee, 2004; Lock et al., 2004) with low levels of LCPUFAs (e.g. EPA and DHA) (Jacobs et al., 2004). Compared with other dietary fat sources, FAs in cow’s milk have a less balanced profile, having lower USFs content and higher short and medium FAs content (Berner, 1993; Kennelly, 1996). The variation in ruminant milk’s FAs composition is due to many reasons, including species, breed, genotype, lactation

stage or environmental factors (Chilliard & Ferlay, 2004; Chilliard et al., 2007). The effect of the lactation stage on milk's FAs profile is related to the magnitude of body fat mobilisation in the first few postnatal weeks (Chilliard et al., 2007). After that, no differences in FA profile were found between mid-and-late lactation (Garnsworthy et al., 2006). Table 1.2 summarises milk's major FAs composition in whole fat milk.

**Table 1.2 Fat composition of whole fat cow's milk in the United Kingdom.**

| **Component** | **Amount per 100 g** |
|---|---|
| Fat (g) | 3.9 |
| SAFs | 2.5 |
| MUFAs | 1.0 |
| PUFAs | 0.1 |
| *Trans* | 0.1 |

Data was taken from the 6th summary edition of The Composition of Foods 2002 (The Dairy Council, 2010).

### 1.4.2 Rumen Lipid Metabolism

Lipid digestion and absorption takes place primarily in the stomach and small intestine of non-ruminants. However, in ruminants, the rumen plays an important role. Different FAs have different effects on rumen fermentation (a process that converts ingested feed into energy sources), which in turn, affects the digestion (Jenkins, 1993). When dietary fat is digested, its transfer to milk fat is influenced by rumen biohydrogenation, absorption, or digestibility and deposition in adipose tissue (Palmquist et al., 1993; Wu et al., 1991). Many studies have used lipid/FAs supplements to enhance milk's FAs profile (Givens & Shingfield, 2006; Chilliard et al., 2000, 2007) and have concluded that the supplement's source and its FAs content

have influenced the effect. In addition, any changes of a specific FAs content will, consequently, affect the other FAs. For example, supplements that increase milk's PUFAs and/or CLA, while decreasing SFAs, will result in an increase of (E)-11-octadecenoic acid (18:1) *trans* (Chilliard et al., 2007; Lock et al., 2005; Roy et al., 2007).

Generally, the quantity of lipids in a ruminants' diet are low, c.1-2% (Kavanagh, 2016). As well as dietary sources, lipids are synthesised in the rumen by bacteria. Ingested lipids are subjected to microbial processing in the rumen, in the form of lipolysis and biohydrogenation (Jenkins, 1980; Palmquist & Harfoot, 1978). These microbial processes convert linoleic acid (LA) to CLA and pave the way to produce CLA isomers (Gorissen et al., 2010).

#### 1.4.2.1 Lipolysis

Lipolysis refers to the process by which dietary lipids are hydrolysed by bacterial lipases to FFAs and glycerol (Buccioni et al., 2012) with little mono or diglycerides (Jenkins, 1993). Then, glycerol is fermented to a volatile, SCFA (Doreau & Ferlay, 1994) which are thereafter absorbed through the ruminal wall, and can be used in the mammary gland for *de novo* synthesis of short-and-medium chain FAs (Buccioni et al., 2012; Mansbridge & Blake, 1997). Released FAs are biohydrogenated and/or contribute to microbial lipids synthesis. SCFAs which result from microbial lipolysis are directed to the blood stream after being absorbed through the ruminal wall (Doreau & Ferlay, 1994). Esterified lipids such as triglycerides, phospholipids and glycolipids are hydrolysed by microbial lipases (e.g. galactosidases and phospholipases): therein, non-esterified fatty acids (NEFAs) are formed (Jenkins, 1993).

#### 1.4.2.2 Biohydrogenation

Biohydrogenation (BH) is a process by which the number of double bonds in USFAs chain are reduced by ruminal microbes to produce SFAs. UFAs have been reported to be more toxic to ruminal microbes than SFAs (Palmquist & Jenkins, 1980): therefore,

BH may have a 'protective role' for microbes against potential toxic effects of UFAs. The process starts with converting the cis-12 double bond to a trans-11 isomer by isomerase, which in turn requires FFAs to have a free carboxyl group to be functional. Therefore, lipolysis is required for BH (Jenkins, 1993). The amount of USFAs that are biohydrogenated is affected by many factors including forage type, concentrate ratio (Kucuk et al., 2001) and can also be reduced by treating dietary lipids chemically or physically, or by adding oilseeds to the diet (Loor et al., 2005; Petit, 2001).

BH produces several intermediates such as FAs isomers. For example, linoleic acid (18:2 n-6) BH results in 18:1 *trans* 11, cis 9 and CLA *trans* 11 (Harvatine et al., 2006). However, these intermediates were produced in low concentrations, and ware included in milk fat at different concentrations (Harvatine et al., 2006).

### 1.4.3 Milk Fat Synthesis

Milk FAs are obtained from two sources: uptake from blood circulation and *de novo* synthesis in mammary cells. *De novo* is synthesised with all CHFAs, all MCFAs up to 12:0, 95% up to 14:0, and 50% of 16:0 in milk. LCFAs are most likely to be obtained from circulating plasma lipids (Chilliard et al., 2000).

In mammary epithelial cells, FAs are synthesised *de novo* from acetate and 3-hydroxy-butyrate in the presence of two key enzymes: acetyl-coenzyme, a carboxylase (ACC; rate limiting enzyme) and fatty acid synthase (FAS; elongation enzyme) (Barber et al., 1997). The acetate is then converted to acetyl CoA, and used in FAs chain elongation, while 3-hydroxy-butyrate is integrated directly after activation to butyryl CoA.

### 1.4.5 Alteration of Milk Fatty Acids Profile by Maternal Dietary Fatty Acid Supplementation

Modifying milk-fat composition through FAs supplements (to enhance FAs) is beneficial to human health and has been well-studied (Chilliard et al., 2000, 2007; Dewhurst et al., 2006; Givens & Shingfield, 2006; Lock & Bauman, 2004; Shingfield et al., 2006).

In ruminants, many studies have investigated the effects of maternal supplementation with canola and sunflower oil (supplements used in this study) on the milk FA profile. In general, these supplements increased MUFAs and decreased SFAs (Hervás et al., 2008; Mir et al., 1999; Okine et al., 2003; Rego et al., 2009; Welter et al., 2016). Table 1.3 summarises the results of these studies.

**Table 1.3 Summary of effects in milk fatty acid profile outcomes in relation to supplementing maternal diet with canola/sunflower oil.**

| Study | Species | Supplement | Outcome |
|---|---|---|---|
| Mir et al. (1999) | Goats | 4/6% canola | Total fat, C18:0, Total CLA |
| | | | MCFAs, C16:0 |
| Okine et al. (2003) | Goats | 4/6% canola | Total fat, MUFAs, C18:0, Total CLA |
| | | | SFAs, MCFAs, C14:0,C16:0, n-3,n-6 |
| Welter et al. (2016) | Cows | 6% canola | MUFAs, C18:0, n-3,n-6 |
| | | | Total fat, PUFAs, MCFAs, C14:0, C16:0 |
| Rego et al. (2009) | Cows | 10% sunflower | MUFAs, C18:0, Total CLA, *trans* fat |
| | | | Total fat, PUFAs, SFAs, MCFAs, C14:0, C16:0, n-3, n-6 |
| Hervás et al. (2008) | Sheep | 6% sunflower | MUFAs, C18:0, Total CLA |
| | | | SFAs, MCFAs, C14:0, C16:0 |

MUFAs = monounsaturated fatty acids, PUFAs = polyunsaturated fatty acids, SFAs = saturated fatty acids, MCFAs = medium chain fatty acids, CLA = conjugated linoleic acid. Red indicates the increase and while blue indicates the reduction compared with controls.

## 1.5 Nutritional Programming

Early-life nutritional programming is a concept introduced by Lucas (1991) which indicates that nutritional practices at critical developmental periods in early life, pre-and-postnatally, can program later life development and health. This has been well-established in human studies of retrospective cohorts in adults (Barker et al., 1989, 1990, 1993; Hales et al., 1991) and infants (Barker et al., 1992; Fall et al., 1995; Martyn et al., 1995; Yajnik et al., 1995) in determining risk of cardiovascular disease, type-2 diabetes and the metabolic syndrome in adulthood. Epidemiological studies have demonstrated these effects (Ericksson et al., 2002 a, b). However, those studies have been criticised for several reasons, including potential confounding factors, lack of adequate analyses to correct confounders, and bias possibility (Huxley et al., 2002; Joseph & Kramer, 1996; Paneth et al., 1996). It is not possible to recreate precise experiments on humans to investigate nutritional programming effects (Gluckman & Hanson, 2004). Indeed, epidemiological studies may be considered a poor tool to study nutritional programming due to the impracticality of using only interventions to explore specific issues, as well as long periods of follow-up (five or six decades by the definition of prospective cohort studies) to consider any possible effects (Langley-Eva et al., 2009). Therefore, animal studies become the only practical way for studying nutritional programming (Langley-Evans et al., 2006, 2009). Animal models, such as rats, mice or sheep, allow investigation into the effects of manipulating maternal diets during different stages of gestation and/or lactation on the development of the foetus and mature offspring. This could be achieved by range of nutritional programs such as: nutrient restriction, excess nutrients and modulating the macro/micro-nutrient intake. My study focuses on the effect of early post-natal nutrition during lactation on programme BAT development. Understanding the relationship between early-life nutrition and later life health is essential to develop appropriate interventions to treat and prevent many diseases and disorders, obesity, and metabolic syndrome.

### 1.5.1 The Effect of Early Life Nutrition on Adult Health

Since the emergence of the Developmental Origins of Health and Disease (DOHaD) hypotheses in the1980s (Barker & Osmond, 1986), numerous studies have focused on the potential relationships between intrauterine and/or early infancy nutrition, and later risk of non-communicable disease. Low birth weight is linked to the risk of death from coronary heart disease, high blood pressure, high circulating clotting factors, type-2 diabetes and metabolic syndrome (Barker et al., 1989, 1990, 1993; Hales et al., 1991). Following low birth weight with rapid 'catch-up growth' in childhood was associated with an increase to the risk of diabetes and coronary heart disease (Ericksson et al. 2002a, b). Sveral studies exhibited the relation between factors that affect foetal growth rates and health in adulthood due to the mothers' diet, suggesting that maternal nutrition is one of the inputs of the programming processes (Roseboom et al., 2001).

### 1.5.2 Early Life Programming of Obesity

The high prevalence of obesity has attracted researchers towards investigating the origins of obesity, as well as the causes and mechanisms underlying it. It is apparent that nutrition in early life can affect later life conditions, including obesity (Roseboom et al., 2001). This programming happens when exposure to stimuli or inhibitors in critical developmental phases (e.g. early postnatal life) alter tissues/organs permanently, causing irreversible alteration in body structure and function (Martin-Gronert & Ozanne, 2013).

Animal models used in experiments have varied to include rodents and large animals with the aim of investigating the possibility of obesity programming through manipulating maternal nutrition. To test a certain approach, specific interventions have been used, such as food intake restriction (Langley-Evans, 2006; Nüsken et al.; 2008), nutrient restriction (Andersen et al.; 2006; Gambling et al., 2003; Stoltzfus, 2003), high or low-fat diets (Khan et al., 2003, 2005; Tamashiro & Moran, 2010), high or low protein diets (Daenzer et al., 2002; Langley-Evans et al., 1996a) and induction

of maternal obesity (Bayol et al., 2007; Samuelsson et al., 2008). Despite the different biological functions between the FAs, most of studies that examine effects of high or low-fat diets have not focused on the maternal diet specifically. My study focused on the potential impact of supplementing the maternal diet with different sources of FAs (e.g. canola and sunflower oils) to aid the development of adipose tissues in early life.

#### 1.5.2.1 Role of Dietary FAs on Adipose Tissue Development and Programming Obesity

In early infancy, the role of dietary fats is not limited to simply being a source of energy, but rather, a source of essential FAs. In humans, milk fat contributes up to 52% of total milk energy (Mace´ et al., 2006) and varies in its macronutrient component between women (Mitoulas et al., 2002). Studying the effect of this variation on infant growth and adiposity is limited to short-term studies due to the practical restrictions, and show no effect on growth rate (Mitoulas et al., 2002). In contrast, it is possible to alter milk's fat quantity and composition by manipulating the maternal diet during lactation (Mace´ et al., 2006). Manipulating the dietary ratio of LA/ALA affects the amount of AA and EPA/DHA in tissues, as AA and EPA/DHA are created from LA and ALA by sequential reactions of desaturation and elongation, during which they compete for the same enzymes. After birth, neonates obtain EFAs (LA, 18:2n-6 and ALA, 18:3n-3) from milk. Although the newborn can use LA and ALA to synthesize small amounts of LC-PUFAs (AA, 20:4n-6; EPA, 20:5n-3 and DHA, 22:6n-3) (Carnielli et al., 1996; Salem et al., 1996), it is insufficient for accreting LC-PUFAs in tissues. Therefore, milk's PUFAs are important for foetal and infant development (Uauy et al., 2003). Historically, n-6 PUFAs have been reported to play an important role in adipose tissue development (Amri et al., 1994; Gaillard et al., 1989) and have a potential impact on childhood obesity in humans (Ailhaud et al., 2006; Mace´ et al., 2006) and animals (Javadi et al., 2004; Massiera et al., 2003; Weiler, 2000).

AA act as a precursor of prostaglandins (PG) which have an important role in cellular processes (Smith, 1989) including promoting adipocyte hyperplasia (increased cell numbers) or hypertrophy (increased cell size) in vitro (Vassaux et al., 1993), and anti-adipogenic roles (Reginato et al., 1998). In mice, a high-fat maternal diet with either high or low LA/ALA ratios (2 or 60 respectively) from mating until weaning (followed by maintaining the same diet for male pups from weaning until 22 weeks of age) led to greater body weight and total fat mass in male offspring compared to those in other groups (Massiera et al., 2003). Interestingly, the body weight and fat mass of prostacyclin-receptor knockout offspring were similar in high and low LA/ALA ratio diet groups, suggesting that prostacyclin signalling promotes adipose tissue development and obesity, which has been attributed to the large amount of LA that is precursor of AA (Massiera et al., 2003). Similarly, high levels of LA in breast milk was associated with high rates of childhood obesity in the US (Ailhaud et al., 2004). On the other hand, other animal studies did not find such effects of LA on adipogenic tissue (Korotkova et al., 2002) or body weight and fat mass (Bourre et al., 1996). In contrast to LA, ALA has an anti-adipogenic role due to its competitive inhibition of Δ6 desaturase: thus, the conversion of LA to AA is downregulating (Hussein et al, 2005)

It is also worth mentioning that in-vivo studies which investigated the potential role of dietary n-3 and n-6 FAs on the development of adipose tissue in early life (and thus, on childhood obesity) had conflicting results (Groh-Wargo et al., 2005; Lauritzen et al., 2005) and need further investigation (Mace´ et al., 2006). Therefore, there is a need for further investigation using animal models as well as epidemiological studies.

## 1.6 Sheep as an Experimental Model for Nutritional Programming

Animal models have played a crucial role in the characterisation of molecular biological mechanisms of obesity and its associated diseases, in discovering the results of nutritional interventions, and understanding the mechanisms underlying these findings. Besides the limited histological samples of humans, it is unethical to test

hypotheses on humans, especially with the possibility of adverse consequences of dietary manipulation in early life. Animals are commonly used as experimental models in certain areas, such as maternal nutrient restriction, placental embolisation, pharmacological intervention, and hypoxia (Martin-Gronert & Ozanne, 2007). Therefore, the choice of model will depend on the research question with respect to resource availability, including funding, equipment, time, and experience.

Although there is no animal model that is very similar to humans, rodents and large mammals are commonly used. However, each model has been criticised for its limitations (McGonigle & Ruggeri, 2014). The advantages of small animals include their low cost and short gestational period, however, they are not ideal models to investigate the impact of nutrition during early life on human health and development in later stages of life. For example, when compared with humans, rodents are immature at birth. The development of their hypothalamic and pancreatic functions occurs postnatally. Moreover, rodents are litter bearing, which reduces offspring birth weight and may affect the amount of nutrients obtained by the foetus in the uterus (Ryan et al., 2002; Röde et al., 2008). As a result, prenatal and postnatal environments are very different between rodents and humans, who tend to bear only one child.

Generally, no animal model summarises human pregnancy perfectly. Nonetheless, sheep are used quite often to investigate the nutritional interactions between mother and foetus (Barry & Anthony, 2008), as they have much in common with humans. They, like humans, are born with a mature hypothalamic–pituitary axis (Clarke et al., 1997) and fully developed organ systems (Symonds et al., 2007). Sheep also have similar weights at birth (c.4.5 kg) (Symonds et al., 2007), even though they bear up to three lambs depending on the breed (Barry & Anthony, 2008). Sheep have a gestational period of 145–150 days, compared to 270 days in humans, while rats have a shorter gestation time of 21–22 days.

However, sheep are not an ideal model for humans. Sheep are ruminants and herbivores: they have a complex digestive system; therefore, their dietary requirements (and glucose utilisation) are different from humans (Barry & Anthony, 2008). Despite this, sheep are the best model for this study to investigate BAT in large mammals, due to the similarity with human babies of BAT abundance around the birth period. In other words, sheep are like humans in thermoregulation in terms of non-shivering thermogenesis (Clarke et al., 1997), while pigs rely on shivering thermogenesis during cold exposure (Symonds & Lomax, 1992).

## 1.7 Overall View of the Thesis: Main Aims and Hypothesis

In this chapter, the potential role of BAT in treating and/or preventing obesity by increasing energy expenditure and nutritional programming of BAT, specifically by early life nutrition was reviewed. As discussed, FAs are known to activate UCP1. Even though milk fat contains approximately 400 different FAs (Månsson, 2008), I was particularly interested in the potential effect of MUFAs, which appear to be present at high levels in the supplements used in this investigation.

The aim of my thesis is to determine whether maternal FA supplementation with a readily available SCFAs (i.e. canola or sunflower oil) modifies the milk FAs profile and thus thermogenic capacity (i.e. UCP1 abundance) of BAT in early postnatal life. Therefore, the growth, development and biological function of different adipose tissue depots were examined together with how these were influenced by the postnatal nutritional environment. The main hypothesis of this study is that the alteration of milk's FAs profile, received in early life, can alter BAT depots development.

The subsequent chapters were designed to test this hypothesis. Figure 1.9 presents the flowchart of this thesis.

- Chapter 3 is an analysis of milk samples from mothers who supplemented with FAs during lactation to alter their milk FAs profile. The aim of chapter 3 was to test the effects of supplementing maternal diet with canola or sunflower oil on milk fatty acid

composition in sheep. This was achieved by analysing milk samples using gas-chromatography (GC) to determine the fatty acid composition in phospholipids.

- Chapter 4 investigates the effects that the modification in the FA composition of maternal milk has on the offspring's adiposity and the expression profile of genes related to BAT. The aim of chapter 4 was to explore the effects of changes in milk fatty acid composion on the offspring with focusing on the potential differences between adipose tissue depots. This was achieved by measuring the body fat composition, plasma metabolite concentrations, organ weights and the potential thermogenic capacity of brown adipose tissue depots in offspring.

- Chapter 5 examines developmental changes within adipocytes in different adipose tissue depots of the offsprings. The aim of chapter 5 was to provide a deeper understanding of the characteristics of adipose depots located in different anatomical locations. This was achieved by analysing a microarray data to identify the most adaptable adipose tissue in response to dietary FA supplementation during lactation. - Chapter 6 assesses the abundance of mitochondrial proteins in different adipose tissue depots. The aim of chapter 6 was to investigate whether the effect of FA composition modification of maternal milk coincided with the changes in the mitochondrial protein abundance in offspring adipose tissue during early life, which may affect the level of energy metabolism within the adipocyte. This was achieved by analyzing adipose tissue mitochondrial proteins using western blotting, histology and immunohistochemistry.

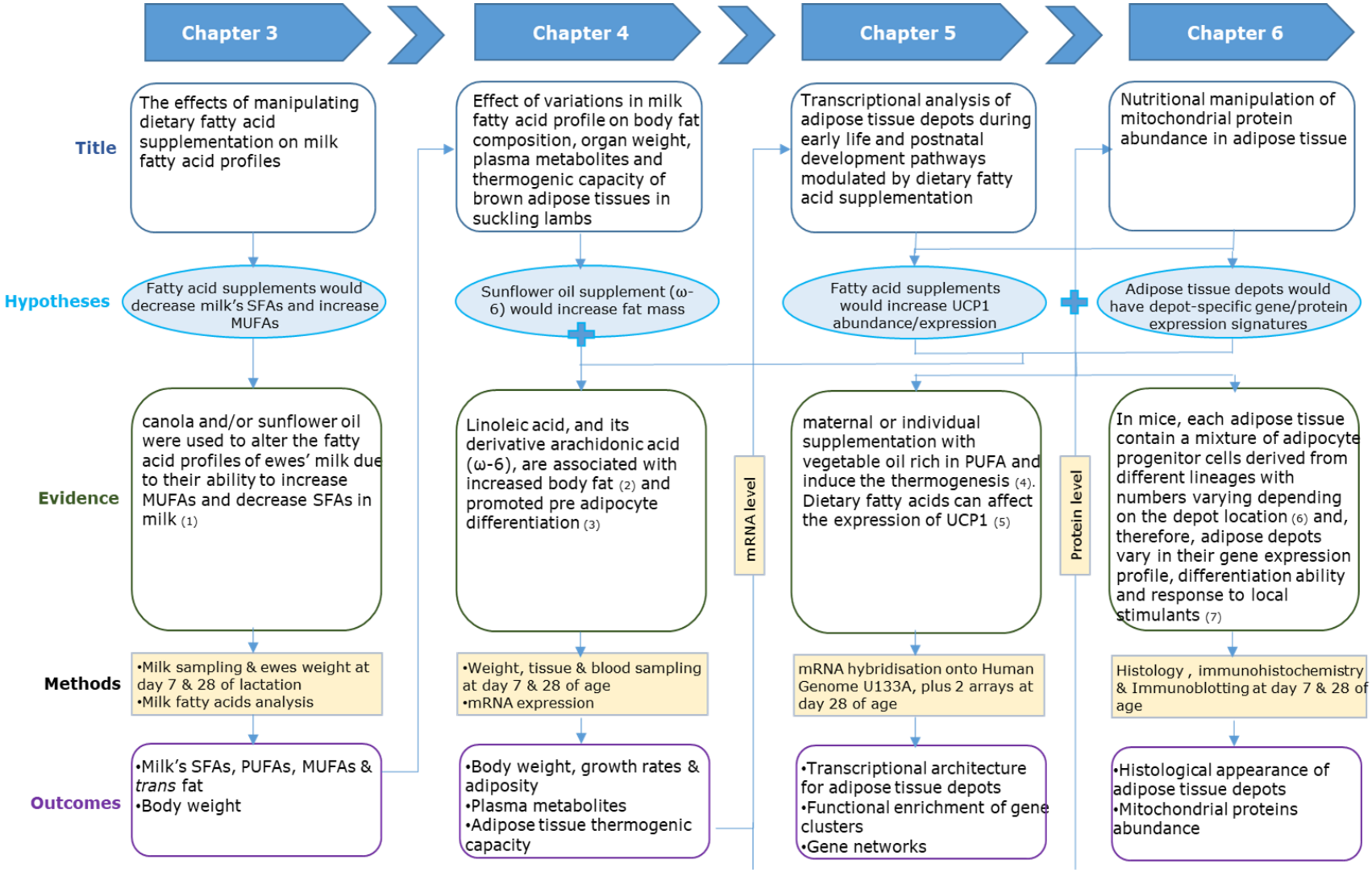



**Figure 1.9 Thesis flowchart diagram.**

(1) Hervás et al., 2008; Okine et al., 2003; Toral et al., 2010; Welter et al., 2016; (2) Javadi et al., 2004; Weiler, 2000; (3) Massiera et al., 2003; (4) Priego et al., 2013; Takeuchi et al., 1995; (5) Fernández-Quintela et al., 2010; Welter et al., 2016; (6) Calderon-Dominguez et al., 2016; Sanchez-Gurmaches et al., 2012; (7) Lee et al., 2017, Vicente-Duenas et al., 2009.

# Chapter 2. Materials and Methodology

In this chapter, the basic scientific theory and the experimental procedures used in this thesis will be explained. In general, the same methodology is used in more than one chapter unless otherwise specified.

## 2.1 Study Protocols and Legislation

The experimental sheep study was conducted in 2013 as part of a research programme to investigate the effect of maternal food supplementation during lactation on the offspring. It was conducted in the Academic Division of Child Health, School of Medicine, University of Nottingham. In line with the principles of reduction in the use of animals, outlined in the 3Rs (NC3Rs, 2011), and in order to explore new hypotheses arising from novel findings in the field since the initiation of the experimental animal work, the tissues generated have been further used in my thesis.

All procedures involving the use of animals in this study were conducted, with UK Home Office approval and in accordance with the UK Animals (Scientific Procedures) Act (1986), by Professor Michael E. Symonds, Dr Viv Perry, Dr Graeme Davies and Dr Mark Birtwistle at the University of Nottingham, Sutton Bonington Campus. These procedures also received ethical approval from the appropriate Ethics Committee of the University of Nottingham.

Safety precautions were taken at all times when working in the laboratory, and all reagents, chemicals and laboratory procedures were assessed and performed in line with the UK Health and Safety Executive's Control of Substances Hazardous to Health regulations (COSHH, SI No. 1657, 1988) and Risk Assessment guidelines. Disposable nitrile gloves and laboratory coats were worn at all times when conducting laboratory procedures. All chemicals were of biology grade. All materials were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Supplier names, details and addresses are given in Appendix 1. Methods were followed as recommended by

the manufacturers or as modified by myself or one of the department technicians, Mr Mark Pope, Mrs Victoria Wilson or Dr Ian Bloor.

All laboratory experiments (except the milk and plasma analyses) were conducted within the Division of Child Health, Obstetrics and Gynaecology, the University of Nottingham and the School of Veterinary Medicine, Sutton Bonington Campus between November 2013 and February 2017, under the supervision of Professor Michael Symonds and Professor Helen Budge.

## 2.2 Study Design

After parturition and until 28 days after delivery, all ewes were fed with either a control diet or a control diet supplemented with 3% canola oil or sunflower oil. The design of the study is shown in Figure 2.1. It should be noted that the numbers of animals detailed in Figure 2.1 represent the total numbers of animals involved my thesis investigation. For different reasons, which will be explained in relevant sections, these numbers vary depending on the performed experiment.

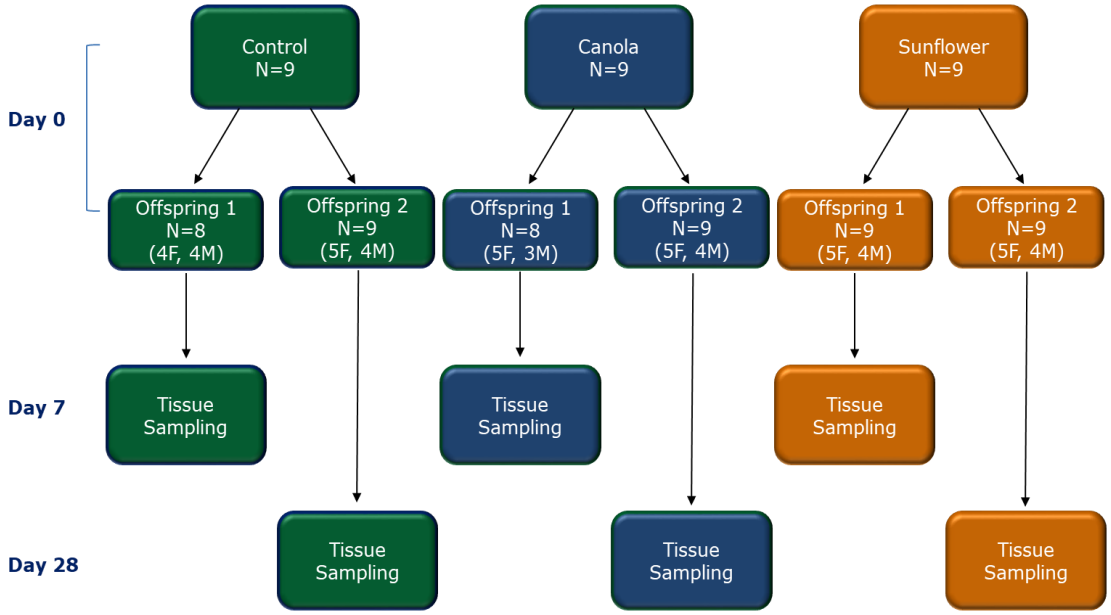



**Figure 2.1 Summary of experimental design.**

Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil. Offspring lambs were mother-fed from birth until tissue sampling day. The day numbers indicate age by days.

### 2.2.1 Animals and Environments

Twenty-eight Bluefaced Leicester ewes were mated with a Swaledale mule ram at the same time of year, so that all lambs were born and sampled as a single cohort.

The ewes were at a commercial farm near Skipton in Yorkshire throughout their pregnancy period. Two weeks before the date of delivery, the ewes were transported to a barn at the Melton Lane Complex on the University of Nottingham's Sutton Bonington campus.

In the barn, the ewes were group-housed until giving birth and were then moved to 2 m x 2 m individual pens with their offspring for the duration of the study.

### 2.2.2 Allocation of Animals to Feeding Groups

The ewes were fed a standard diet until they give birth normally at term (21 weeks). Of the 28 ewes, 23 gave birth to twins and 5 to triplets, making an initial total of 61

lambs: 35 females and 26 males. Immediately after delivery, the ewes were allocated to one of three feeding groups: the standard or 'control' diet (n = 10); the standard diet supplemented with 3% canola oil (n = 9); or the standard diet supplemented with 3% sunflower oil (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.

One female lamb was stillborn, and another one died within 24 hours of birth, leaving 33 surviving female lambs. One male lamb was excluded from the study due to stunted growth, making 25 the total number of male lambs. The five ewes which gave birth to triplets were allocated to the control group. From each of the five sets of triplets, one lamb was separated from its mother and sampled on the day after birth (1M, 4F), leaving each mother raising two siblings until sampling day. This meant that there were 53 total remaining lambs (29 female and 24 male).

### 2.2.3 Animal Feeding

#### 2.2.3.1 Ewes

The pregnant ewes were fed a standard diet until delivery, consisting of 2 kg hay and 1 kg standard commercial concentrate (Ewe 18 Mix; Manor Farm Feeds, Oakham, Leicestershire, UK). The concentrate was prepared for pregnant and lactating ewes and was sufficient to fulfil recommended dietary requirements (Alderman & Cottrill, 1993). After giving birth, the ewes were allocated to one of three feeding groups:

- the control group, fed on 2 kg of hay along with 1 kg of standard commercial concentrate;
- the canola group, fed on 2 kg of hay along with 1 kg of supplemented concentrate with 3% canola oil; and
- the sunflower group, fed on 2 kg of hay along with 1 kg of supplemented concentrate with 3% sunflower oil.

Ewes allocated to the canola and sunflower groups had double the amount of total fats and oils compared with the controls. The constituents of the concentrates are shown in Table 2.1. All groups had free access to water.

**Table 2.1 Constituents of concentrate feed by dietary group.**

| 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 |

#### 2.2.3.2 Lambs

All lambs were milk-fed by their mothers from birth until they were tissue sampled at either day 7 or day 28.

### 2.2.4 Anthropometric Measurements

#### 2.2.4.1 Body weight measurements

The lambs' body weight (kg) was measured on their day of birth, and on days 7 and 28, when both the lambs' and ewes' body weights were taken.

#### 2.2.4.2 Blood sampling

Blood samples were taken from all the lambs just before they were tissue sampled (day 7 or 28). Up to 20 ml of blood was taken from the jugular vein with a 10 ml syringe in two extractions. The blood was relocated into a 10 ml ethylenediaminetetraacetic acid (EDTA) tube (Vacutainer; BD, Plymouth, Devon, UK) and into a 9 ml lithium heparin tube (S-Monovette; Sarstedt, Nümbrecht, Germany).

These were immediately cooled in ice and then, within 20 minutes, the tubes were centrifuged at 4 °C and 1200 g for 15 minutes. The plasma was extracted and allocated into 2 ml cryovials, which were stored at –80 °C. Metabolite and hormone analysis was done by Professor Duane Keisler (University of Missouri, USA), Dr Nigel Kendall (School of Veterinary Medicine, University of Nottingham) and Dr Rachel Woods (Department of Academic Child Health, University of Nottingham).

#### 2.2.4.3 Milk sampling

Milk samples were collected from the ewes on days 7 and 28 and were then transferred into two sterile 15 ml tubes (Greiner Bio-One, Gloucester, UK). The samples were stored at –80 °C. Analysis of the milk's fatty acids was done by Tamas Marosvolgyi (The University of Pécs, Hungary) using high-resolution capillary gas-liquid chromatography as described by Kovács et al. (2005).

#### 2.2.4.4 Tissue sampling

One lamb from each ewe was sampled at around 7 (± 1) days of age and the other at 28 (± 3) days of age. Lambs were randomly selected for sampling time points in sex-matched pairs, but cases of male-female pair selection were affected by the need to maintain the sex balance within each age group as much as possible.

Lambs were humanely euthanised with an overdose of barbiturate (200 mg $kg^{-1}$ body weight of pentobarbital sodium; Euthatal; Merial Animal Health, Harlow, Essex, UK) injected into the jugular vein at their allotted sampling age. The lambs were then dissected, and specific tissues and organs were excised and weighed. Samples were snap-frozen in liquid nitrogen before being stored at −80 °C until later analysis. Samples from perirenal, epicardial, omental and sternal adipose tissue were also preserved in 10% formalin (3.45% formaldehyde, 0.9% sodium chloride) for subsequent histological analysis. Dissections were carried out by Professor Michael Symonds, Dr Mark Birtwistle and Dr Viv Perry and were assisted by Dr Graeme Davies.

## 2.3 Histology

### 2.3.1 The Principle of the Technique

Histology is the study of the microscopic structure of thin sections of tissue to distinguish different biological structures after these have been appropriately stained. In order to prevent tissue degradation, tissues are chemically fixed in formalin prior to histology, which irreversibly binds to amino acids, to preserve cellular structures and prevent protein degradation (Werner, 2000). Following tissue fixation, each sample is encased in a holding matrix, to enable sample sectioning without affecting the structural composition. Paraffin wax is a common matrix used in combination with light microscopy. It safely permeates the sample and preserves its morphological structure. However, it is necessary first to dehydrate the tissue, usually by immersion in ethanol and xylene, before embedding it in the wax, as it is immiscible with water. Once the tissue paraffin block solidifies, thin sections can be sliced using a sledge microtome and can then be transferred onto a glass slide and dried. To allow visualisation and/or differential identification of microscopic structures, a wide range of stains are used in special techniques to add colour or enhance the colour of certain types of cellular structures differently from other components located in the examination area. Finally, the samples are examined under a light and/or digital microscope.

### 2.3.2 Sample Preparation and Histological Tissue Processing

Random sections of sternal, perirenal and epicardial tissue samples, which were stored at room temperature, were taken from paraformaldehyde-fixed tissues from each age group (3.45% v/v formaldehyde in 0.9% w/v sodium chloride/distilled water (Fisher Scientific, Loughborough, Leicestershire, UK) saline solution). Segments of each sample (c. 1 $cm^2$) were loaded into a Histosette II (Simport Scientific, Quebec, Canada) 30 mm × 25 mm × 5 mm cassette and were processed through increasing concentrations of ethanol from 75% to 100% in order to dehydrate the tissue (six 1-hour stages) at 20 °C, followed by ethanol clearing with xylene (Fisher Scientific,

Loughborough, Leicestershire, UK) (three 45-minute stages) at 20 °C and were then immersed in paraffin wax (Tissue-Tek Embedding Wax; Sakura Finetek, Alphen aan den Rijn, Netherlands) (three 45-minute stages) at 60 °C using a Shandon Excelsior™ advanced tissue processor (Thermo Shandon Ltd, Runcorn, Cheshire, UK) (Table 2.2). Sections were embedded at random orientations in cassettes containing paraffin wax using a Tissue-Tek III Embedding Center (Sakura Finetek) and were then allowed to solidify overnight to ensure that each sample segment was fully fixed.

When the tissue samples had been blocked in the paraffin, each block was sectioned at a thickness of 5 µm using a sledge microtome (AS200; Anglia Scientific, Cambridge, UK). Each slice was rinsed in 70% ethanol and floated in a 40 °C water bath to stretch it out by surface tension. Sections were transferred to Superfrost™ Plus glass microscope slides (Menzel-Gläser; Gerhard Menzel, Braunschweig, Germany) and were dried on a heat rack at 50 °C for one hour to evaporate all water before they were dried in a drying oven at 37 °C for 24 hours.

In general, about 10 slides were prepared from different levels throughout each tissue block for this assay and for subsequent procedures which were conducted in this study.

**Table 2.2 Protocol for processing tissues.**

| Step | Chemical | Time |
|---|---|---|
| 1 | 75% Ethanol | 60 min |
| 2 | 90% Ethanol | 60 min |
| 3 | 95% Ethanol | 60 min |
| 4 | 100% Ethanol | 60 min |
| 5 | 100% Ethanol | 60 min |
| 6 | 100% Ethanol | 60 min |
| 7 | Xylene | 45 min |
| 8 | Xylene | 45 min |
| 9 | Xylene | 45 min |
| 10 | Paraffin wax | 45 min |
| 11 | Paraffin wax | 45 min |
| 12 | Paraffin wax | 45 min |
| **Total** | | 10.5 hours |

### 2.3.3 Tissue Staining with Haematoxylin and Eosin (H&E)

The H&E stain is a combination of haematoxylin and eosin, making up one of the principal, most commonly used stains in histology. Haematoxylin is a basic aluminium salt dye with a positive charge, which consists of haematein dye and aluminium ions ($Al^{+3}$); it is used with a 'mordant' (i.e. aluminium salt), and makes it act as a basic dye. Once the aluminium salt mordant is added, haematoxylin is oxidised to haematein by mercuric oxide, and it binds to acidic structures in tissue, such as nucleic acids, which are negatively charged (i.e. DNA in the nucleus and RNA in ribosomes and the rough endoplasmic reticulum). In other words, aluminium salts binds to the tissue, and then haematoxylin binds to the aluminium salts. Harris' haematoxylin, one of three main aluminium haematoxylin solutions, is commonly used to stain nuclei purple before examination under a microscope. It is a regressive dye which is 'blued off' with a weak alkali (Kiernan, 2002; Bancroft & Marilyn, 2007),

meaning that the tissue is overstained with blue, and then a differentiator is used to remove the excess dye (Bancroft & Gamble, 2008). In comparison, Eosin Yellowish is negatively charged and acts as an acidic dye which stains basic structures red or pink; these structures include most proteins in the cytoplasm which are positively charged (i.e. erythrocytes, cytosol, collagen and muscle fibres) (Bancroft & Marilyn, 2007). Differentiation of the basic formations occurs through washing with water.

#### 2.3.3.1 H&E staining procedure

Sections were stained with H&E using a standard protocol. One slide from each sample was placed into a slide rack. First, the slides were dewaxed by immersion in a xylene container (2 × 3 minutes). Secondly, the slides were rehydrated by immersion in a series of ethanol baths beginning with 100% ethanol (2 × 3 minutes) and then in 70% ethanol/distilled water for 3 minutes, and they were then rinsed in distilled water for 3 minutes. Thirdly, the sections were nuclear-stained in a container of Harris' haematoxylin (VWR Ltd, Lutterworth, UK) for 5 minutes. Excess dye was removed by rinsing in a continual flow of tap water for 5 minutes. Differentiation was performed by immersion in an acid-alcohol (1% concentration hydrochloric acid in 70% ethanol) solution for 15 seconds and by bluing in alkaline Scott's tap water (a 0.2% sodium bicarbonate and 20% magnesium sulphate distilled water solution) for 1 minute to revert the haematoxylin stain. The slides were washed in a continual flow of tap water for 5 minutes and were transferred to a 1% Eosin Yellowish (VWR Ltd) counter-stain for 5 minutes, followed by washing in tap water for 90 seconds to remove any excess stain, which allowed the differentiation of eosinophilic structures. Next, the sections were dehydrated back through immersion in a series of 100% ethanol baths (2 × 2 minutes) and were cleared in xylene for 2 and 3 minutes, sequentially (Table 2.3). Finally, the sections were mounted with glass coverslips (VWR) using DPX mounting medium (Fisher Scientific, Loughborough, Leicestershire, UK) and were left to dry overnight.

**Table 2.3 Protocol for H&E Staining.**

| Step | Chemical | Time |
|---|---|---|
| 1 | Xylene | 3 min |
| 2 | Xylene | 3 min |
| 3 | Absolute Alcohol | 3 min |
| 4 | Absolute Alcohol | 3 min |
| 5 | 70% IMS | 3 min |
| 6 | Distilled water | 3 min |
| 7 | Harris' haematoxylin | 5 min |
| 8 | Tap water | 5 min |
| 9 | HCI in 70% alcohol | 15 sec |
| 10 | Scott's tap water | 1 min |
| 11 | Tap water | 5 min |
| 12 | 1% Eosin | 5 min |
| 13 | Tap water | 90 sec |
| 14 | Absolute Alcohol | 2 min |
| 15 | Absolute Alcohol | 2 min |
| 16 | Xylene | 2 min |
| 17 | Xylene | 3 min |

### 2.3.3.2 Adipocyte H&E analysis

The H&E-stained slides were visualised through a Nikon Eclipse 90i microscope (Nikon UK Limited, Surrey, UK) and were imaged by Volocity© v5.2.0 image software (Perkin Elmer, Massachusetts, USA). The slides contain about 1 $cm^2$ of tissue were scanned and visualised through a Leica DRM microscope (Leica Microsystems, Milton Keynes, UK) at 4× and 20× magnification and were photographed for analysis using a Retiga-2000R CCD digital camera (QImaging, Surrey, BC, Canada) at 20× magnification.

Images were analysed using Image-Pro Premier software (Media Cybernetics, Rockville, MD, USA). To determine the change in white space with age and/or diet, that represented lipid droplets of white adipocytes within brown depots, the percentage of positively stained cells in the whole tissue section was estimated using

Objective Imaging's Surveyor software (Objective Imaging Ltd, Cambridge, UK). To calculate the mean volume of the white adipocytes, a line was drawn around the periphery of white adipocyte areas using a 'free hand' tool and avoiding any areas of tissue with vessels or large areas of brown adipocytes.

Settings were refined to detect the whole white space in the tissue without including small white areas in a brown adipocyte. Refinements included the size of the white space which was to be counted and the minimum and maximum white space detection limits. Table 2.4 shows the values used to define the white adipocytes. To ensure sufficient detection for white adipocytes areas, different values were trailed, and visual examination was performed.

The total area of white space for each area was measured by the software, and then the cell numbers within each area were counted using a 'tagging' tool and were recorded. The mean volume of white adipocyte was calculated by dividing the total volume of white space within the areas by the total number of adipocytes.

**Table 2.4 Settings applied in Image-pro Premier for measuring mean cell volume and mean number of white adipocytes.**

| | | |
|---|---|---|
| **HSI Threshold** | Start | 212 |
| | End | 255 |
| **Range (µm)** | Start | 98 |
| | End | 100,000* |

The HSI threshold used to determine the appropriate intensity of white to enable an accurate measure of total white space. The range was set to ensure that only areas of white spaces likely to be white adipocytes were counted.

* The end value was chosen, as it was larger than any single white adipocyte, to ensure that any large white spaces which were unlikely to be a cell were not counted.

## 2.3.4 Immunohistochemistry

### 2.3.4.1 Principle of the technique

Immunohistochemistry (IHC) is a technique used to detect and determine the morphological localisation and distribution of a specific antigen (i.e. a protein of interest) in a histological section; it exploits antibody-target antigen interactions, which produce a signal during formation. The visualisation of these signals can be achieved by using a conjugated signalling marker, such as an enzyme, a fluorescent dye or a conjugated antibody with a direct or indirect method of staining (Mason & Sammons, 1978; Ramos-Vara, 2005). In direct staining, a primary antibody conjugate is used to bind with the target antigen, which results in the production of a signal. In indirect staining, primary and secondary antibody conjugate complexes are used, which leads to more specificity compared to direct staining (Ramos-Vara, 2005) (Figure 2.2).

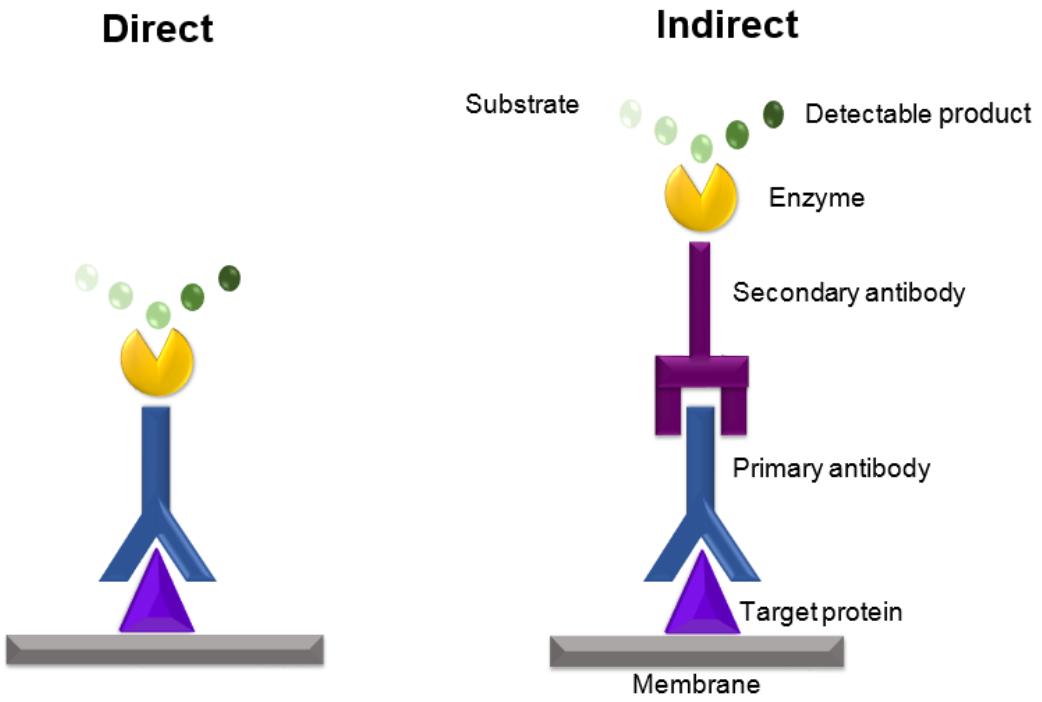



**Figure 2.2 Antibody action in direct and indirect immunostaining methods.**

In direct IHC, specific primary antibody conjugates are used to bind to the target antigen, whereas in indirect IHC, secondary antibody conjugates are used to bind to the primary antibody to form an antigen-primary-secondary antibody complex. In both methods, signals (e.g. colour development) are produced for measurement.

The most common enzyme used in the indirect IHC method is horseradish peroxidase (HRP). In brief, in the presence of a chromogenic substance called 3,3'-diaminobenzidine (DAB), HRP develops a brown precipitate through catalytic conversion (Graham & Karnovsky, 1966). As all tissues are fixed initially in formalin, it is important to dewax and rehydrate them in order to allow access to the tissues by all the reagents; this can be achieved by following the same steps described in the H&E staining procedure in section 2.3.3.1. However, many antigen sites can be masked by the cross-linking of proteins as a result of formalin fixation, which may lead to non-specific binding and high staining. Therefore, all of these protein cross-links need to be broken and all hidden antigen sites should be unmasked by treating with a heat-

induced epitope retrieval (HIER) step (Shi et al., 1991), which usually involves heating with citrate buffer.

#### 2.3.4.2 IHC procedure

IHC analysis was performed in order to determine the level of brown adipocytes in the sternal, perirenal and epicardial tissues. UCP1 primary antibody raised in rabbit against sheep prepared 'in house' (Schermer et al., 1996) was used in this study at a dilution factor of 1:750 (Bispham et al., 2003).

Positive and negative controls were used in each experiment. A positive control is a sample of tissue known to contain the target protein and subject to the same processing as the samples. In my experiments, a one-day-old sample of ovine perirenal adipose tissue was used as a positive control. A negative control is a sample of tissue which is subject to the same processing as the samples except for being exposed to the antibody. In my experiments, a one-day-old sample of ovine perirenal adipose tissue and four samples at least from the tissue to be tested were used as negative controls. To ensure the specificity of the primary antibody, a sample of kidney, which was known not to contain the target protein, was used as an additional control and underwent the same treatment as the samples. Figure 2.3 shows examples of positive and negative controls.

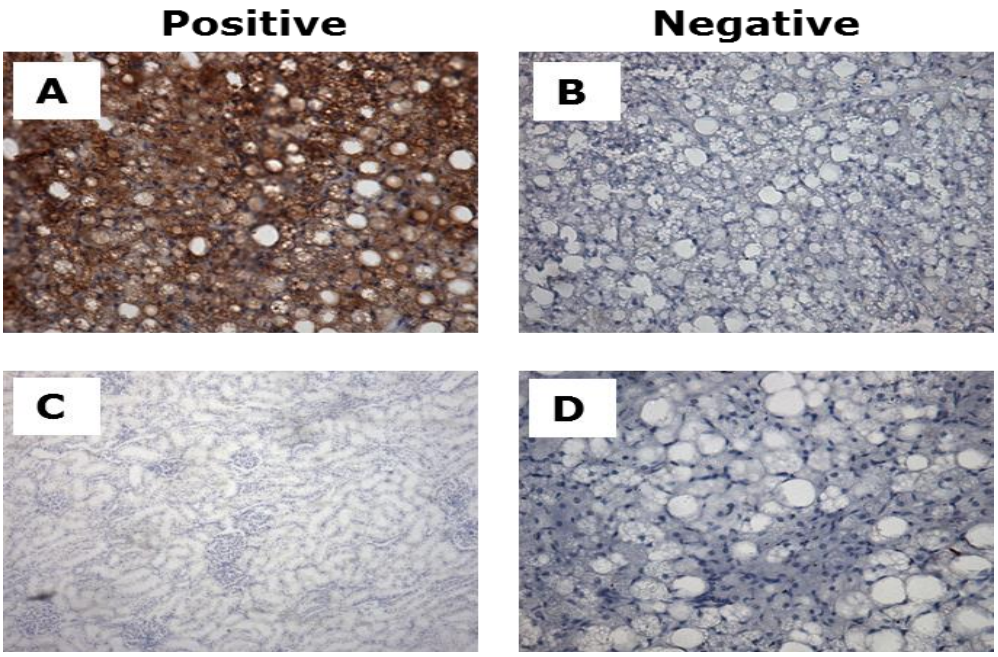



**Figure 2.3 Example of (A, C) positive and (B, D) negative controls.**

Positive and negative slides were included on each run on the BondMax. The positive slides were processed the same way as all other slides; they included (A) a one-day sample of ovine perirenal adipose tissue which was known to contain UCP1 and (C) a one-day sample of kidney which was known not to contain UCP1. The negative slides were subject to the same processing as all other slides except for being exposed to UCP1; these included (B) a one-day sample of ovine perirenal adipose tissue and (D) a representative image of a one-day sample of sternal adipose tissue.

One slide per animal of perirenal, epicardial and sternal adipose tissue from each dietary group at two time points as well as the positive and negative controls were labelled, placed in a slide rack, loaded into a Leica BondMax™ IHC slide processor (Leica Microsystems) and then run on an automated software programme (Vision Biosystems Bond version 3.4A, Leica Biosystems, Newcastle, UK) using Bond Polymer Refine Detection reagent (Leica Biosystems, Newcastle, UK).

The slides were first dewaxed three times with Bond Dewax Solution (which contains a solvent-based solution) and were then rinsed three times with 100% ethanol for one minute each. After that, epitopes were retrieved four times with Bond Epitope Retrieval Solution 1 (a citrate-based buffer), were rinsed twice at ambient temperature, were incubated for 20 minutes at 98 °C and were incubated for 12

minutes at room temperature. After the washing steps, peroxidase blocking was carried out for 5 minutes at room temperature using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Next, all samples (except the negative control) were exposed for 15 minutes to 150 µl of home-set primary antibody against UCP1 (Schermer et al, 1996), which was diluted to 1 in 750 in Bond Primary Antibody Diluent (containing TBS, surfactant, protein stabiliser and 0.35% ProClin 950). The slides were washed twice and were then incubated in 150 µl of secondary antibody (HRP-conjugated secondary anti-mouse and rabbit antibody polymer) for 8 minutes and were rewashed. Thereafter, the slides were incubated in 150 µl substrate Mixed 3,3'-DAB Refine for 10 minutes (until brown precipitate had developed) followed by 3 rinses with distilled water. Following this, nuclear staining was performed with 150 µl of Harris' haematoxylin for 5 minutes followed by a final washing.

After each stage of the BondMax programme, the 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. The slides were dehydrated through ethanol and cleared in xylene before they were mounted with glass coverslips (VWR Ltd) using DPX mounting medium (Fisher Scientific, Loughborough, Leicestershire, UK) and were dried overnight.

In total, at least 10 slides per animal from perirenal, epicardial and sternal adipose tissue were stained for further analysis.

#### 2.3.4.3 Adipocyte analysis

The slides were visualised at 4×, 10× and 20× magnification using Leica DM RB and Nikon Eclipse 90i microscopes (Nikon UK Limited, Surrey, UK) with a charge-coupled device and high-speed colour camera (Micropublisher 3.3RTV; QImaging, Surrey, BC, Canada). Images were captured using Volocity quantification software (v6.1.1, Improvision Ltd, Coventry, UK). For IHC quantification, the slides were scanned using NDP NanoZoomer digital pathology (C9600-02, Hamamatsu Photonics Limited, Hertfordshire, UK), which imaged the whole slide and generated a large representative digital image 'digital slide'; then these enormous digital files were viewed and/or

analysed using NDP.view2 Viewing software U12388-01 version 2.6.13 (Hamamatsu Photonics Limited, Hertfordshire, UK).

Images were prepared for analysis using NDP.view2 software. Twenty 0.1 mm² boxes were distributed over the whole tissue section scan image, avoiding the outer edge where the tissue is usually folded, causing non-specific staining. Each box was cropped and saved as an individual image (Figure 2.4).

The analysis of IHC was performed using the free open access software ImageJ (http://rsbweb.nih.gov/ij/), following a similar manual procedure to that of analysing the IHC slides.

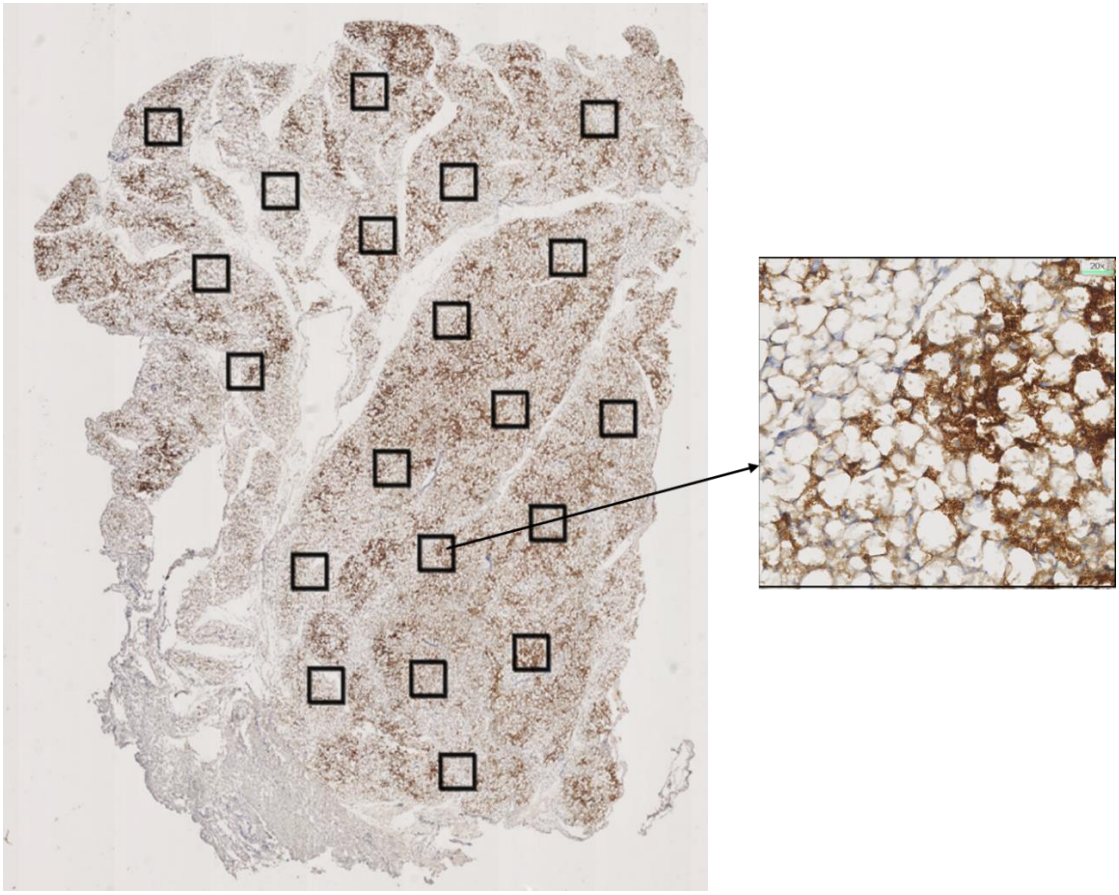


**Figure 2.4 Illustrative image to illustrate the selection of areas for IHC analysis.**

Twenty 0.1 mm² boxes were randomly distributed across the tissue, avoiding the outer edge and tear areas.

#### 2.3.4.3.1 Quantification of adipocyte size and number

First, each image was calibrated to allow the programme to recognize the sample size based on the number of pixels and the magnification of the microscope; this was done using the 'Set Scale' tool, and the settings were maintained for all subsequent image analyses through selecting the 'Global' box. The backgrounds of the images were removed based on a 'rolling ball' algorithm (Sternberg, 1983) by selecting the 'Subtract Background' tool to clarify the image.

In order to avoid over-/under-detection of the presence of the stain, threshold ranges were refined by me. Table 2.5 shows the values used to define the UCP1 stain threshold.

**Table 2.5 Settings applied in ImageJ for measuring mean cell size of brown adipocytes.**

| | | |
|---|---|---|
| **Hue Threshold** | Start | 202 |
| | End | 255 |

The hue threshold was used to determine the appropriate intensity of brown to enable an accurate measure of the total brown stain.

The total volume area of adipocytes in field of view for each image was measured by the software and was recorded in an Excel spreadsheet. Following this, to increase the accuracy of the measure and facilitate cell counting, each cell membrane was enhanced using the 'Dilate' tool. After that, the number of cells in field of view was counted manually by using a tool allowing the placement of the count number in the centre of the cell; see Figure 2.5. Ten photos/slides per sample were analysed before the average of cell size or count was calculated.

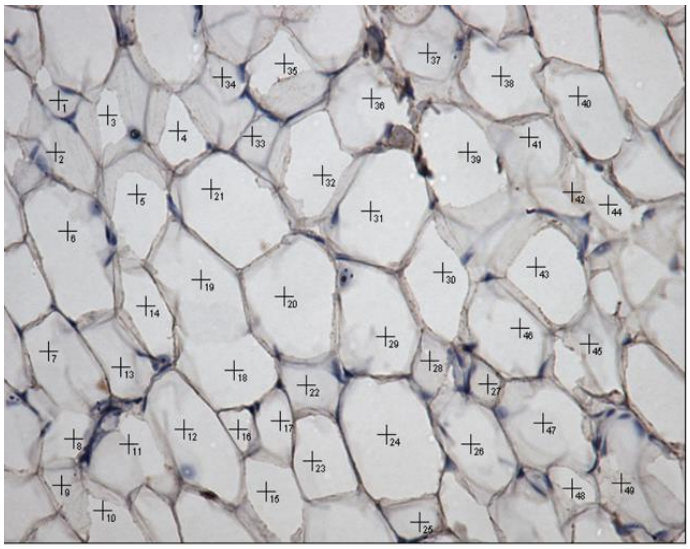


**Figure 2.5 Example of cell counting.**

The number of cells was counted manually by using a tool allowing the placement of the count number in the centre of the cell. Only the whole cells were included.

#### 2.3.4.3.2 Quantification of UCP1

First, all images were converted to an 8-bit grayscale image using the 'Image-Type-8-bit' tool to minimize the risk of manipulation in assessing the stained area. To count stained cells, positively and negatively stained areas were made distinctive by placing marks of different colours. The total dark area (including cell membranes) was automatically measured by the software and recorded. Positive UCP1 staining was assessed by setting the threshold manually, using the 'Image–Adjust–Threshold' tool, to the values described in Table 2.5. The 'Limit to Threshold' option was selected to ensure that all the grey level measurements were measured rather than the entire image. The total UCP1-stained area for each image was measured by the software and recorded. From this, a relative total percentage of UCP1 staining was calculated by dividing the total dark area by the total UCP1-stained area. An arbitrary unit was chosen as a measurement unit, as the amount of tissue on each slide was similar but not identical. This allowed me to compare depots/groups for relative UCP1 staining.

As no slides were labelled with the treatment code, this was a blinded procedure which eliminated the investigator bias during analysis.

## 2.4 Tissue Protein Analysis (Western Blotting)

Western blotting (also known as protein immunoblotting) is a technique using antibodies to detect and analyse the size and expression of a protein of interest in biological cell lysate samples (Burnette, 1981; Jensen, 2012). The method consists of several steps. First, tissue samples are homogenised and lysed to extract the proteins of the sample, and then the protein concentration is measured using bicinchoninic acid (BCA). The samples are then separated by heating with sodium dodecyl sulphate (SDS), an ionic detergent which gives the proteins a negative charge (Jensen, 2012), and with β–mercaptoethanol to prevent the formation of a disulphide bond linking the amino acids. After this, the samples are loaded onto a 1-D polyacrylamide gel and are separated by electrophoresis. The principle of separation is that negatively charged particles (proteins treated with SDS) migrate towards the positive electrode according to their size. In order to ensure that the protein which is separated is the protein of interest, a protein marker ladder is loaded onto the gel alongside the samples, allowing the verification of the sample size. To visualise the sample movement, loading dye is added to the samples.

Once the proteins have been separated, they can be transferred from the gel matrix to an electrostatic nitrocellulose membrane using semi-dry electroblotting methods.

A 'sandwich' of buffer-soaked filter paper stuffed with the gel is laid between two membranes and placed into a semi-dry blotter. Then, a small electric charge is passed through the ‘sandwich’, resulting in the transfer of proteins from the gel to the membrane. After the protein transfer, the membrane is stained with a reversible protein dye to ensure that the protein has been transferred completely and evenly.

The protein detection mechanism is similar to the one described for the IHC (2.3.4.1). The membranes are incubated in a blocking solution containing a different protein to stop any non-specific binding of antibodies before incubation in the specific primary and secondary antibody dilutions, resulting in building a target antigen-antibody protein complex. The secondary antibody used in my study was labelled with horseradish peroxidase (HRP), which, when exposed to luminol, releases a detectable

signal which is directly proportional to the abundance of the target protein. The chemiluminescent signal was detected by exposure with a CCD camera, and quantification was determined by analysing the protein band density using densitometry software.

### 2.4.1 Western Blot Mitochondrial Preparation Procedure

For each sample, 200 mg of sternal and perirenal tissue was cut and placed in a Dispomix® tube and was homogenised with 1 ml of mitochondrial homogenisation buffer (10 mM Tris solution, 250 mM sucrose solution, 1 mM EDTA solution, pH 7.4) using a Dispomix® closed system homogeniser, to separate out the cells from the tissue. The tubes were then centrifuged for 1 minute at 3000 g (3861 rpm) at room temperature in order to concentrate the homogenate at the bottom of the Dispomix® tubes; then all the homogenate from each sample tube was transferred into three separate 1.5 ml Eppendorf tubes labelled with the corresponding sample numbers. To ensure that there was no remaining tissue, the samples in each Dispomix® tube were washed with 100 µl of homogenisation buffer, which then was added to the corresponding Eppendorf tubes; the samples were further homogenised manually using a hand-held Potter-Elvehjem homogeniser in order to lyse the cells and release the mitochondria. The homogenate for each sample was recombined into one Eppendorf tube and centrifuged for 10 minutes at 800 g (2744 rpm) at 4 °C to concentrate the fat at the top of the tube and the cellular debris at the bottom. The supernatant was transferred into a new 1.5 ml Eppendorf tube and centrifuged for 60 minutes at 13,000 g (11,063 rpm) at 4 °C in order to concentrate the mitochondria at the bottom of the tube. Finally, the supernatant was removed from each tube, and 100 µl of homogenisation buffer was added and pipette mixed to resuspend the mitochondrial pellet.

### 2.4.2 Determination of Protein Concentration (BCA Assay)

A BCA assay was used to determine the mitochondrial protein concentration depending on the colorimetric reactions between proteins, BCA and copper sulphate.

By the end of the assay, the colour changed from green to purple in proportion to the protein amount present in the sample. Following this, the absorbance could be measured at 570 nm, and a standard curve, made up of known concentrations, was created, which allowed the unknown concentrations to be referenced.

To do this assay, 50 ml of 'reagent A' was made up, using 1% bicinchoninic acid, 2% sodium carbonate, 0.16% sodium tartrate and 0.4% sodium hydroxide. This was brought up to volume with distilled water and brought to pH 11.25 using 10% sodium bicarbonate. Then, 50 ml of 'reagent B' was made up, using 4% copper sulphate brought up to volume with distilled water. Then, 'reagent C' was made up by mixing reagents A and B, to the ratio of 100 reagent A: 2 reagent B, and was stored at 4°C.

The samples were diluted by 1:20 in 0.9% saline using 2.5 µl of each sample, to make up a total volume of 50 µl. Then, standards were prepared in a 1 mg/ml stock solution of bovine serum albumin (BSA) in 0.9% saline using 8 concentrations of BSA ranging from 1.0 to 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 mg/ml. Table 2.6 shows the volumes used to make up the standards.

**Table 2.6 Volumes used to make up standards.**

| Standard concentration (mg/ml) | µl BSA from 1mg/ml stock | µl Saline (0.09%) |
|---|---|---|
| 1.0 | 100 | 0 |
| 0.8 | 80 | 20 |
| 0.6 | 60 | 40 |
| 0.4 | 40 | 60 |
| 0.2 | 20 | 80 |
| 0.1 | 10 | 90 |
| 0.05 | 5 | 95 |
| 0 | 0 | 100 |

After this, 10 µl of saline (negative control), standard s or sample was pipetted in triplicate to a 96-well plate, and then 200 µl of reagent C was added to each well. The plate was covered with a film and was incubated in an orbital shaker for 30 minutes at 37 °C in order to allow colour development. Then, the plate was placed into a plate reader, and the absorbance was measured at 570 nm. A 5% coefficient of variance between triplicates was accepted, and a 20x multiplication factor was applied to all results to calculate the dilution factor.

### 2.4.3 Western Blot Polyacrylamide Gel Electrophoresis Procedure

#### 2.4.3.1 Preparation of gel for electrophoresis

A 12% polyacrylamide-resolving gel was made as described in Table 2.7. The gel was then cast carefully in an 18 cm by 16 cm Hoefer SE600 gel chamber (Hoefer Inc, Massachusetts, USA), using a syringe to avoid the introduction of air bubbles to the gel, allowing a 3–4 cm space for the stacking gel and loading comb. Then, about 6 ml of water-saturated butanol was poured on the top of the gel to level it and prevent it from drying out, and the gel was allowed to set at room temperature for one hour. After that, the water-saturated butanol was poured off and the gel washed off with distilled water. Then, a 3% stacking gel was made as described in Table 2.8 and was poured carefully above the resolving gel to nearly fill the chamber but allow space on the top to insert a 0.75 mm loading comb used to form the sample wells. The gel was left to polymerise at room temperature for 1 hour.

**Table 2.7 Reagents and quantities used to prepare the resolving gel.**

| Reagent | Quantity |
|---|---|
| Distilled water | 8 ml |
| 30% polyacrylamide mix (37:5:1) | 6.6 ml |
| 1.5 M Tris (pH 8.8) | 5 ml |
| 10% Sodium dodecyl sulphate (SDS) (50 g SDS/500 ML water) | 200 µl |
| 10% Ammonium persulphate (APS) (1 g APS/10 ml water) | 200 µl |
| Tetramethylethylenediamine (TEMED) | 8 µl |

**Table 2.8 Reagents and quantities used to prepare the stacking gel.**

| Reagent | Quantity |
|---|---|
| Distilled water | 6.8 ml |
| 30% polyacrylamide mix (37:5:1) | 1.7 ml |
| 1.5 M Tris (pH 6.8) | 1.25 ml |
| 10% Sodium dodecyl sulphate (SDS) (50 g SDS/500 ML water) | 100 µl |
| 10% Ammonium persulphate (APS) (1 g APS/10 ml water) | 100 µl |
| Tetramethylethylenediamine (TEMED) | 10 µl |

### 2.4.3.2 Preparation of samples for electrophoresis

From each protein sample, 20 µl at a concentration of 4.2 µg/µl was diluted in a sterile Eppendorf tube using the equation:

Ci x Vi = Cf x Vf

(Ci) initial protein concentration (Cf) final concentration

(Vi) initial protein volume (Vf) final volume

The final sample concentration (Cf) for these 20 µl was 4.2 µg/µl, because the amount of protein in the total volume of 84 µl of my mix was 84 µg:

Cf = 84 µg/20 µl = 4.2 µg/µl

The initial protein volume (Vi) to reach the final volume (Vf) of 20 µl was calculated as follows:

Vi = (4.2 µg/µl * 20 µl)/initial protein concentration (which was measured as mentioned in section 2.4.1).

The samples were brought up to 84 µl, as described in Table 2.9. Then, the samples were incubated in a water bath for 10 minutes at 95 °C to dissociate the proteins and were immediately placed on ice after removal from the water bath.

**Table 2.9 Reagents and quantities used to prepare the protein mix.**

| Reagent | Quantity |
|---|---|
| Protein association buffer (containing 50 mM Tris, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol and distilled water pH 6.8) | 50 µl |
| Glycerol-bromophenol blue loading dye (containing 0.01 g bromophenol blue, 14.9 g sodium hydroxide and 23.5 ml distilled water) | 14 µl |

Of the diluted protein samples, 20 µl at the desired concentration was added to make up 84 µl of protein mix.

#### 2.4.3.3 Loading the gel and running electrophoresis

After the loading comb was removed from the gel, the wells were rinsed with distilled water, and the electrophoresis chambers were filled with 1X running buffer (5 mM Tris, 50 mM glycine (Fisher Scientific, Loughborough, Leicestershire, UK), 2% SDS and distilled water). Twenty µl of each sample, the negative (sheep liver), and positive controls (one-day-old lamb perirenal tissue) and 10 µl of the protein molecular weight marker ladder (Thermo Scientific) were randomly loaded in duplicate into the wells, and the order was carefully recorded. Any empty wells were loaded with 20 µl loading dye to ensure even running during electrophoresis. A reference sample was added to all gels to allow result calibration. In order to ensure even loading of protein and to

complement total-protein estimates, Ponceau S has beeen added as a loading control (see Appendix 2). Ponceau S is a reversible total-protein stain which has been reported to be a possible alternative to multiple loading controls (Janes, 2016). Then, the gel chamber was carefully placed into the electrophoresis chambers. The electrophoresis apparatus was assembled and connected to a power pack (Power PAC300, BIO-RAD, USA), and the gel was run for approximately one hour at 150 V until the dye progressed to 1.5 cm away from the bottom of the gel.

#### 2.4.3.4 Protein transfer procedure

Once the dye moved a suitable distance, the electrophoresis chambers were disassembled and the gel carefully removed. The stacking gel was cut, and the resolving gel was placed directly onto the nitrocellulose membrane (GE Healthcare, UK) after it was cut to the same size as the gel. Then, four sheets of 1 mm Whatman blotting paper (Scientific Laboratory Supplies, UK) were cut to the same size as the gel and soaked in Towbin buffer (containing 25 nM Tris, 192 mM glycine, 20% v/v methanol and distilled water, pH 8.3). Two sheets of blotting paper were then placed on either side of the gel and membrane to create a 'sandwich' which was carefully squeezed with a test tube to remove any air bubbles. After the gel sandwich was placed between two foam pads, it was placed into the blotting apparatus, ensuring that the nitrocellulose membrane was toward the anode (Figure 2.6). The unit was filled with blotting buffer, and the cooling bath was turned on and the proteins transferred at 80 V for one hour.

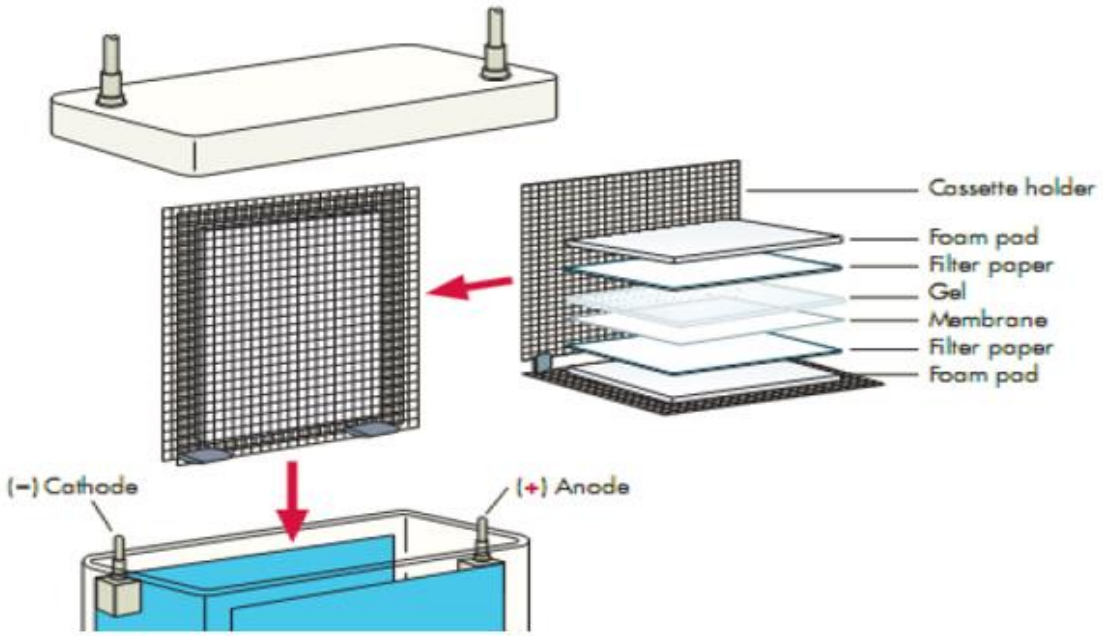



**Figure 2.6 Illustration of the assembly of gel and membrane for Western blot protein transfer.**

Two sheets of filter paper placed on either side of the gel and membrane to create a 'sandwich' which placed between two foam pads. The sandwich placed into a cassette holder before placed into the blotting apparatus. The membrane placed toward the anode (+). The unit was filled with blotting buffer, and the cooling bath turned on and the proteins transferred at 80 V for one hour.

### 2.4.3.5 Staining procedure

After protein transfer, the nitrocellulose membrane was immersed in Tris-buffered saline with Tween® 20 (TTBS) and was then soaked for 10 minutes in 100 ml 1:10 Ponceau S red stain solution (containing 0.2 g Ponceau S, 3 g trichloroacetic acid (Fisher Scientific, Loughborough, Leicestershire, UK), 3 g sulphosalicylic acid (Acros Organics, NJ, USA) and distilled water) in order to visualise the protein band positions and ensure even loading. The molecular weight markers from the protein ladder were marked using an antigen/antibody pen (for rabbit primary antibodies, Alpha Diagnostic, TX, USA), and then the Ponceau S stain was removed from the membrane with distilled water and TTBS. The membrane was then blocked overnight at 4 °C in blocking solution (10% w/v milk powder (Marvel, Premier Foods, Spalding, UK) in

TTBS), with care being taken to ensure complete powder dissolution to avoid any spots on the membrane.

### 2.4.4 Western Blot Protein Detection Procedure

The membrane was removed from the fridge and blocked at room temperature for an additional 30 minutes on a shaker before being rinsed twice with TTBS. Next, the membrane was incubated for two hours at room temperature on a shaker with 15 ml primary antibody, diluted to the appropriate dilution. Details of the primary antibody concentrations used in this investigation are listed in Table 2.10. The membrane was then washed on a shaker three times in 50 ml of TTBS, for 10 minutes each time, at room temperature. Following this, the membrane was incubated for two hours, on a shaker at room temperature, with 10 ml of secondary antibody (polyclonal swine anti-rabbit immunoglobulins/HRP (P0217, Dako Ltd., Ely, UK) at a 1:2,000 dilution in a 3% (w/v) milk solution). After this, the membrane was rinsed twice with TTBS and washed in TTBS four times for 15 minutes each time, at room temperature on a shaker. An additional wash in TTBS for 30 minutes was performed on the membrane, at room temperature on a shaker, before it was soaked in TBS for a further 45 minutes at room temperature.

**Table 2.10 Primary antibodies used in Western blot protein detection.**

| Antibody | Source | Dilution |
|---|---|---|
| Uncoupling Protein (UCP) 1 | raised in rabbit against sheep prepared 'in house' * (Schermer et al., 1996) | 1 in 10000 |
| Voltage-dependent anion channels (VDAC) | an ovine-specific antibody prepared 'in house' * | 1 in 2000 |
| Cytochrome c | ('Santa Cruz', Santa Cruz, CA, USA) | 1 in 1000 |

* based on the method of Schermer et al. (1996)

For chemiluminescent visualisation, a solution of 6 ml of hydrogen peroxide and 6 ml of luminol reagent (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Watford, Hertfordshire, UK) was applied directly onto the membrane and was incubated at room temperature for 5 minutes. Following this, the membrane was dried using blotting paper and was carefully covered in cling film, so as not to include air bubbles. Then, the positive control wall was masked and the protein bands were imaged using a luminescent image analyser with one-minute exposure under visible light (EPI) and chemiluminescence.

Protein band densities were analysed using the Aida Image Analyzer v.4.15 (Raytek Scientific Ltd., Sheffield, UK). In this evaluation, the abundance of polypeptide in the sample is reflected in the density of the bands after deducting the intensity of the background staining.

## 2.5 Tissue Gene Expression Analysis

All the following experiments used for the gene expression analysis were performed on the lambs' sternal, perirenal and epicardial adipose tissue with precautions taken to minimise the contamination of reagents, instruments and samples. To do this, the work benches and instruments were cleaned with RNase ZAP® (Ambion, CA, USA), RNAase ZAP® (Ambion, California, USA) and 70% ethanol. All equipment used was autoclaved, and the pipette tips were filtered. Sterile nuclease-free water (Ambion, Inc, UK) was used for all dilutions and reagent preparations. Samples were transferred from –80 °C freezers on ice prior to processing, were dissected to the required amount and were placed in labelled containers. Experimental work was performed on ice where necessary.

### 2.5.1 Ribonucleic Acid Extraction

In order to extract the total RNA from tissue, an RNA extraction kit can be used. The required samples are lysed and homogenised in a monophase solution containing phenol and guanidine thiocyanate, which causes the denaturing and dissolution of all proteins and the inhibition of any RNAase activity (Chomczynski, 1993) to ensure the

obtaining of intact RNA by the end of the process. The addition and then incubation with chloroform followed by centrifugation results in the separation of the mixture to three phase layers: the RNA dissolved in the top clear aqueous phase, the DNA dissolved in the middle phase and the proteins dissolved in the bottom phase (Chomczynski & Sacchi, 1987; Chomczynski, 1993). After that, the upper aqueous RNA solution is separated, and then ethanol is added to provide appropriate binding conditions to the RNA centrifuge column where the total RNA (≥100 µg) binds to the membrane. A series of washings with ethanol and guanidium salt-based buffers is carried out to remove any contaminants before the final elution of the RNA with nuclease-free water.

It is necessary to determine the purity and concentration of the extracted samples where it is possible, to contain impurities of the protein. One method to determine this is by spectrophotometer at a wavelength of 260 nm, where nucleic acids absorb UV light, and thus, RNA concentration can be estimated through relative wavelength absorption. In addition, as protein absorbs UV light at a wavelength of 280 nm, sample contamination with protein can be measured by a ratio of absorption between wavelengths 260 nm and 280 nm (Glasel, 1995), with a recommended ratio between 1.8 and 2.0 (Wilson & Walker, 2010).

#### 2.5.1.1 RNA extraction procedure

The tissues' RNA was extracted using the RNeasy plus kit (Qiagen, Crawley, UK). Seven-day-old (c. 250 mg) or 28-day-old (c. 1.0 g) tissue samples were individually thawed in a Dispomix® tube with 1 ml of Tri-reagent (Sigma-Aldrich) and were homogenised at 3000 rpm in a Dispomix® closed system homogeniser (Thistle Scientific Ltd. Glasgow, UK). An additional 0.5 ml and 1 ml of Tri-reagent, respectively, was added to the 7- and 28-day-old homogenised samples, and then they were incubated for 10 minutes at room temperature to allow the breakdown of the lipid. The homogenate was vortexed 5 times over 30 seconds and was centrifuged at 3220 g for 1 minute at 4 °C. Then, the homogenate — the lower phase of the sample — was pipetted into a sterile 2 ml Eppendorf tube, and 200–500 µl of

chloroform (depending on the tissue sample age) was added, vortex mixed and incubated for 10 minutes at room temperature in order to allow phase separation.

After incubation, the samples were centrifuged at 12,000 g for 15 minutes at 4 °C. The top aqueous phase of each sample was carefully pipetted, with care being taken not to disturb the lower phase, and was then added into a genomic deoxyribonucleic acid (gDNA) eliminator column (included in the kit) and centrifuged for 1 minute at room temperature at 8000 g. After that, 700 µl of ethanol was added to each sample and vortex mixed. Next, 700 µl at a time of each sample was transferred into an RNeasy spin column (included in the kit) and was then centrifuged for 15 seconds at room temperature at 8000 g. This was repeated until the whole sample had been through the spin column, discarding any flow-through, with all RNA being kept in the column.

Next, 700 µl of RW1 (premade buffer, included in the kit) was added to each column and centrifuged for 15 seconds at room temperature at 8000 g, discarding the flow-through. Following this, 500 µl of RPE buffer (premade buffer, included in the kit) was added and centrifuged for 15 seconds at room temperature at 8000 g, discarding the flow-through. An additional 500 µl of RPE buffer was added and centrifuged for 2 minutes at room temperature at 8000 g. The RNeasy spin columns were then transferred to a sterile 2 ml collection tube and centrifuged for 1 minute at room temperature at 8000 g to remove all remaining RPE buffer. Then, each RNeasy spin column was transferred to a sterile, labelled 1.5 ml Eppendorf tube, and 40 µl of nuclease water was added to each column, which was centrifuged for 1 minute at room temperature at 8000 g. This final step was repeated to increase the concentrations of RNA yield.

#### 2.5.1.2 Determination of RNA concentration purity and integrity

The extracted RNA concentration (ng/µl) and purity (260:280 nm and 260:230 nm ratios) were measured using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, DE, USA) with a 260:280 nm ratio of c. 2.0, where a 260:230 nm ratio of c. 2.0–2.2 indicates a good RNA quality (Manchester, 1996). RNA obtained from 28-day-

old adipose tissue was further analysed for RNA integrity by the Agilent 2100 Bioanalyzer using the 2100 Bioanalyzer Eukaryote total RNA kit (Agilent Technologies, USA). In this assay, the RNA samples were separated by electrophoresis on a micro-fabricated chip and were then detected by laser-induced fluorescence detection in the presence of an RNA ladder as a mass and size standard during electrophoresis. The ladder allowed the estimation of the RNA band sizes, and then the integrity of the RNA was assessed by visualisation of the 18S and 28S ribosomal RNA bands (Figure 2.7). The RNA integrity number (RIN) for each sample was calculated using the 2100 Expert Software version 2.6 (Agilent Technologies, USA), based on the shape of the curve in the electropherogram. Samples which had an RIN >7 were selected for microarray analysis.

In order to avoid a recurrence of the freeze and thawing which causes RNA degradation (Wilson & Walker, 2010), 10 µl of each RNA sample was aliquoted and diluted to 1 µg/µl to normalise the samples and make them ready for the reverse transcriptase-polymerase chain reaction (RT-PCR); they were then stored in labelled tubes at –20 °C until further analysis. The residual RNA was stored at –80 °C for use when needed.

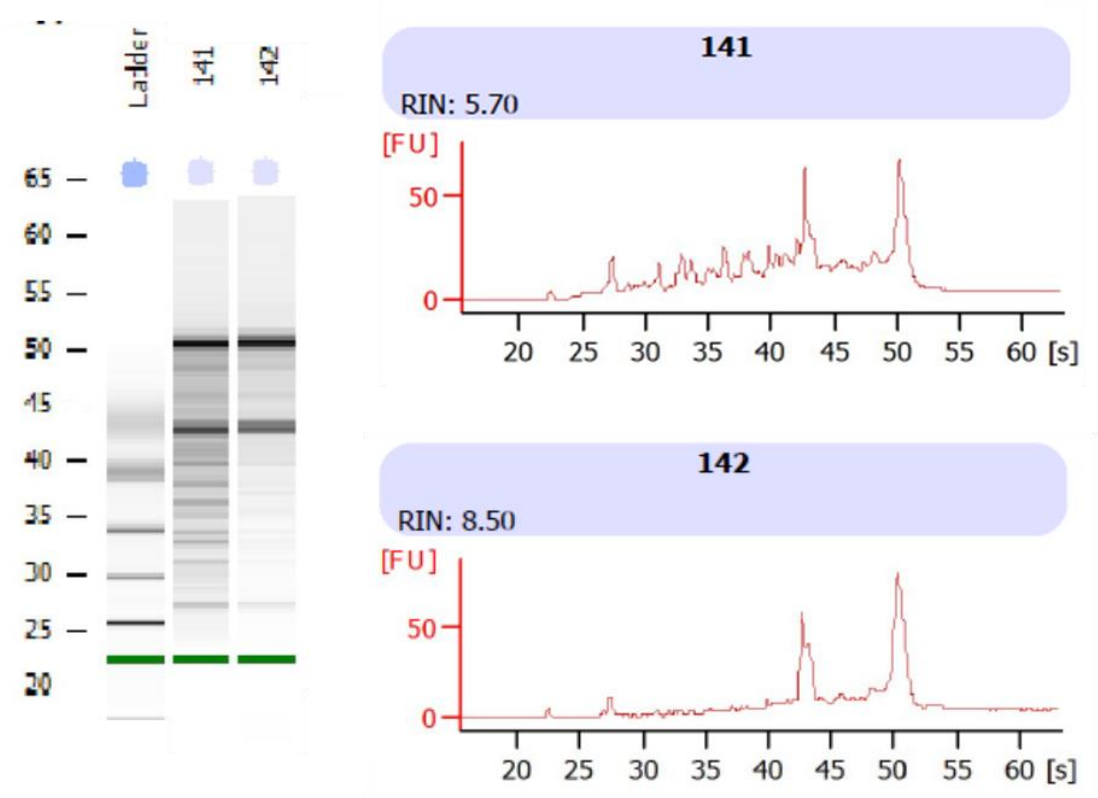



**Figure 2.7 Chromatograms of micro-capillary electrophoresis.**

Two RNA samples showing different degrees of integrity. Sample 141 is degraded (RIN = 5.70), as indicated by multiple peaks in the electropherogram, and an increase in the fluorescent signal indicates the destruction of dye intercalation sites. Sample 142 had an RNA integrity number (RIN) of 8.50, and the electropherogram is typical for high-quality RNA, showing a clearly visible 28/18S ribosomal RNA peak ratio.

### 2.5.2 Polymerase Chain Reaction (PCR)

A polymerase chain reaction (PCR) allows the isolation, amplification and subsequent analysis of a specific region of a little-known DNA sequence using a pair of complimentary oligonucleotide sequences ('primer', forward and reverse) in an enzymatic reaction (Butler, 2001). This reaction contains the target DNA, primers, thermostable enzyme (Taq Polymerase), deoxyribonucleoside triphosphates (dNTPs) and appropriate pH and ion buffer. The amplification happens through repeated cycles of the heat denaturation of the target DNA's double-stranded leads to separate the double helix strand. Then, the primers anneal to the 3' terminus of each strand, and

the extension of these annealed primers occurs by enzymatic reaction. Each cycle doubles the product of the DNA, resulting in an exponential amplification of the DNA of approximately $2^n$ (n = number of cycles) (Saiki et al., 1988). This is illustrated in (Figure 2.8).

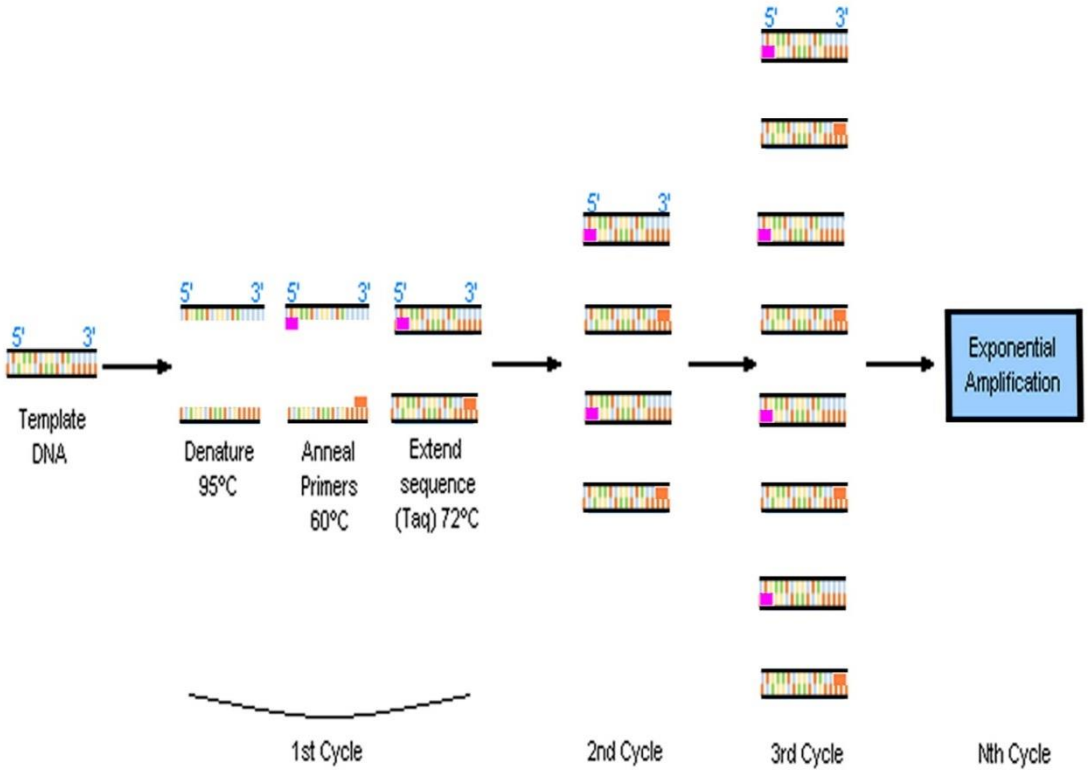



**Figure 2.8 Representation of the amplification which occurs during the polymerase chain reaction.**

Template DNA is amplified exponentially in an enzymatic reaction of cycles by complimentary oligonucleotide primers.

Primers should be specific to the region of interest, as elongation and amplification will only occur on the DNA sequence to which the complimentary primers have annealed. Because of this, primer design is an important feature when working with PCR analysis. When the primers are poorly designed, the incorrect sequence could be amplified or the product yield decreased either by interaction between primer pairs to form primer dimers or by different annealing and melting temperatures which are affected by the primer sequence and length (Butler, 2001). General parameter guidelines for optimal PCR primer design are listed in Table 2.11.

**Table 2.11 General parameter guidelines for optimal PCR primer design (Butler, 2001).**

| Parameter | Optimal Values |
|---|---|
| Primer Length | 18–30 bases |
| Melting Temperature (Tm) | 55–72 °C |
| Tm difference between forward and reverse primer set | < 5 °C |
| Guanine/cytosine (GC) content (%) | 40–60% |
| Self-complimentarity (hairpin structures) | < 3 continuous bases |
| Complimentarity to other primer (primer dimers) | < 3 continuous bases |
| Distance between two primers on target sequence | <2000 bases apart |
| Identical continuous bases | < 4 continuous bases |

### 2.5.3 Reverse Transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) is a process which can transcribe single-stranded RNA into double-stranded complementary deoxyribonucleic acid (cDNA) by using a reverse transcription enzyme and a DNA primer. This produces cDNA strands which can undergo classical PCR, as it works by amplifying a double-stranded molecule (DNA).

The reaction is initiated by binding a DNA primer to single-stranded mRNA, and the sequence is extended by a reverse transcriptase enzyme, creating cDNA (Figure 2.9) ready to be amplified in classical PCR.

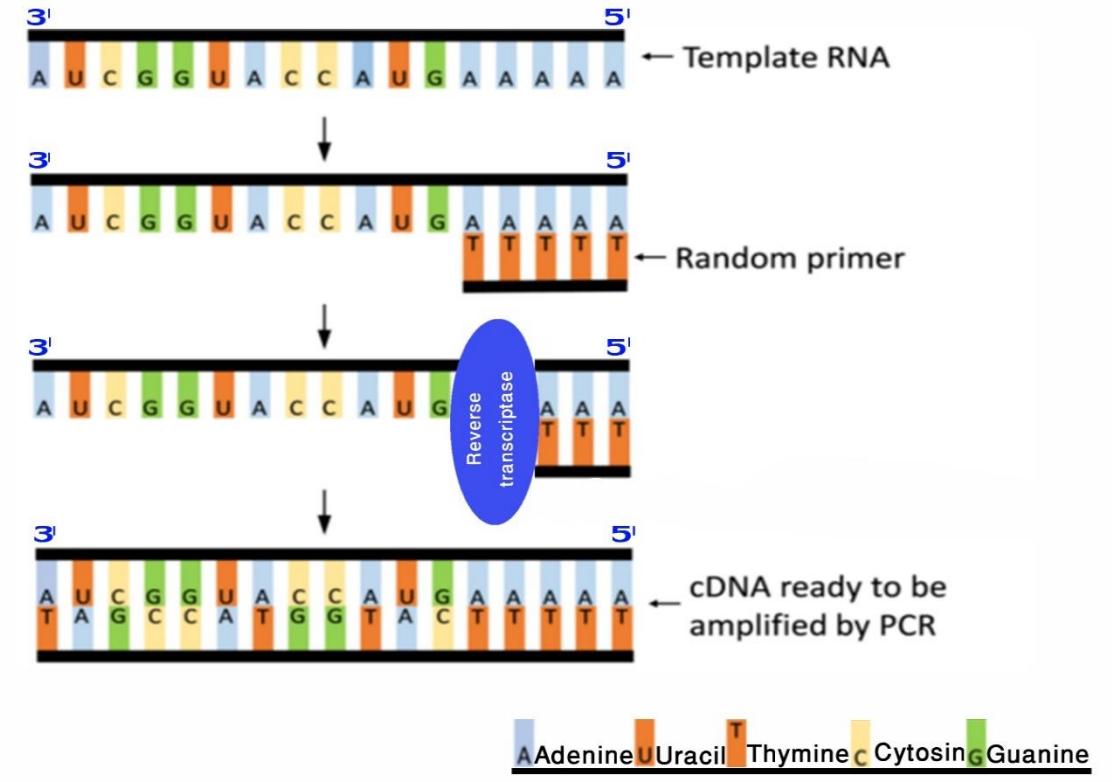



**Figure 2.9 Diagram of reverse transcriptase-polymerase chain reaction.**

A DNA primer sequence anneals to template mRNA, where elongation and transcription occurs by enzymatic reaction, creating double-stranded cDNA which can be amplified by polymerase chain reaction (PCR).

### 2.5.3.1 RT-PCR procedure

An RT-PCR procedure was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Warrington, UK).

RNA samples were thawed on ice. In order to ensure transcription efficiency, two 0.2 ml RNase-free Eppendorf tubes were labelled for each sample, one for the reverse transcription (RT+) reaction and one for a non-reverse transcription enzyme control (RT-).

For each sample, 1.5 µl of a 1 µg/µl sample of RNA was mixed with 10 µl of 2X RT buffer and 1 µl of 20X RT Enzyme Mix (included in the kit), and the volume was brought up to 20 µl by adding nuclease-free water.

Two negative controls were also prepared, a no-template control (NTC) (containing the RT buffer, the RT enzyme mix and nuclease-free water instead of the RNA sample) and a no-reverse transcriptase control (NRTC) (containing the RNA sample, the RT buffer and nuclease-free water instead of the RT enzyme). Details about the preparation of the samples and controls are listed in Table 2.12.

**Table 2.12 Details of the reagents and quantities used to prepare the samples; no template control (NTC) and no reverse transcriptase control (NTRC).**

| | **Sample (µl)** | **NTC (µl)** | **NRTC (µl)** |
|---|---|---|---|
| **RNA sample** | 9 | 0 | 9 |
| **RT buffer** | 10 | 10 | 10 |
| **RT enzyme mix** | 1 | 1 | 0 |
| **Nuclease-free water** | 0 | 9 | 1 |
| **Total volume** | 20 | 20 | 20 |

The samples tubes were then centrifuged for 1 minute at room temperature at 4000 g and were placed into in the Techne Touchgene Gradient thermal cycler (Techne Incorporate, New Jersey, USA) where the 'RT RNA 2 cDNA' programme (described in Table 2.13) was run. After this, the cDNA samples were diluted by 1 in 10 with nuclease-free water, and both the template and the diluted cDNA samples were stored at –20°C until further analysis.

**Table 2.13 Details of the 'RT RNA 2 cDNA' cycle for polymerase chain reaction.**

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

## 2.5.4 Quantitative Polymerase Chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) is used to accurately measure the quantity of a gene of interest present within a sample. Using this technology, DNA or mRNA sequence detection and quantification can take place simultaneously and in real-time in vitro while dispensing of additional procedures such as gel electrophoresis, which reduces the expense, required time and the risk of contamination (Reischl & Kochanowski, 1995). Moreover, qPCR eliminates the need for using hazardous fluorescent detection reagents, such as ethidium bromide.

In a qPCR run, a DNA sequence is amplified and bound to a non-hazardous dye which fluoresces, and the PCR product is then detected and quantified by measuring the intensity of the fluorescent signal, which positively correlates to the quantity of DNA (Bustin, 2004). As the fluorescent dyes have a level of background fluorescence, the qPCR will only detect the signal when a certain threshold of fluorescence above this background level is crossed. This threshold is referred to as the cycle threshold (Ct) or crossing point (Cp), and the greater the number of templates at the beginning of the reaction, the fewer the number of cycles required to cross this threshold. This means that the Ct can be used as a parameter to quantify the initial amount of target template in the sample (Bustin, 2004).

One of the commonly used fluorescent dyes is SYBR Green, which fluoresces upon binding to double-stranded DNA and can be used with any gene, making it both economical and time-efficient. Because of this, though, it is non-specific and can bind

to any DNA present in the sample, and this non-specific binding is something that should be taken into consideration if this will affect the achievement of the intended goal. On the other hand, non-specificity was particularly advantageous for this investigation because the sheep genome, the animal model used in this study, is not fully annotated, and therefore modelling specific investigation is challenging. In order to achieve meaningful results with the non-specific SYBR Green, it is important to eliminate any contaminating genomic DNA (gDNA) and to design the primers such that the risk of primer dimer formation is as low as possible. Additionally, any non-specific amplifications with SYBR Green can be detected by evaluating the melt curves, wherein a single peak indicates the specificity of the product which has been amplified.

#### 2.5.4.1 The Melt Curve

The qPCR analyses the melt curve at the end of the reaction by quantifying the fluorescent signal. By the completion of qPCR amplification, the temperature increases from 60°C to 95°C, which causes the dissociation of the double-stranded cDNA at a given melting temperature to release the SYBR green (Ponchel et al., 2003; Denman & McSweeney, 2005). A single specific cDNA will dissociate at a specific temperature, producing a single sharp peak (Figure 2.10). Thus, broader peaks or curves that display multiple peaks indicate the presence of non-specific products.

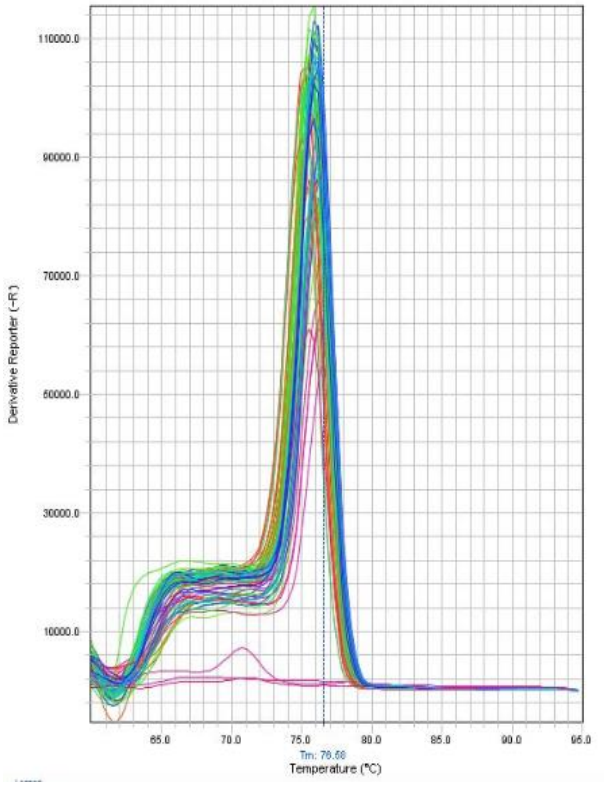



**Figure 2.10 Representative melt curve from qPCR.**

A single peak in the melt curve of all the samples indicates the specificity of the product amplified. The pink coloured lines represent the negative controls.

### 2.5.4.2 The Standard Curve

Because the PCR instrument software plots the exponential amplification graphically as a sigmoidal curve, a comparison of the linear section of the curve can be performed between samples run within the same experiment (Figure 2.11). Therefore, the cDNA concentration can be quantified by the standard curve method, where plotting the log concentration (ng/µl) of the set of known standard serial dilutions against cycle number in which the Ct was crossed can be used to calculate a straight line equation (Higuchi et al. 1993; Arya et al. 2005) (Figure 2.12). From this, the efficiency of the reaction (E) and correlation coefficient ($R^2$) can be determined, and these values provide an additional verification of the experiment.

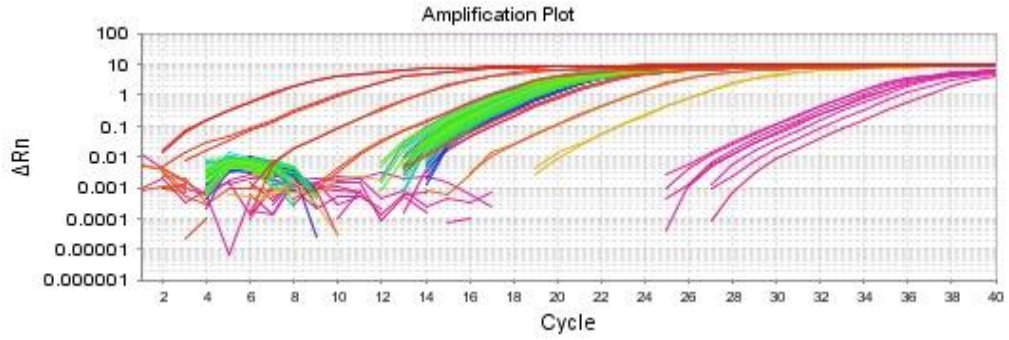



**Figure 2.11 Representative amplification plot from qPCR.**

Fluorescent signal (ΔRn) is increased exponentially with amplification. Red lines represent external standards, green are unknown samples and pink represent negative controls.

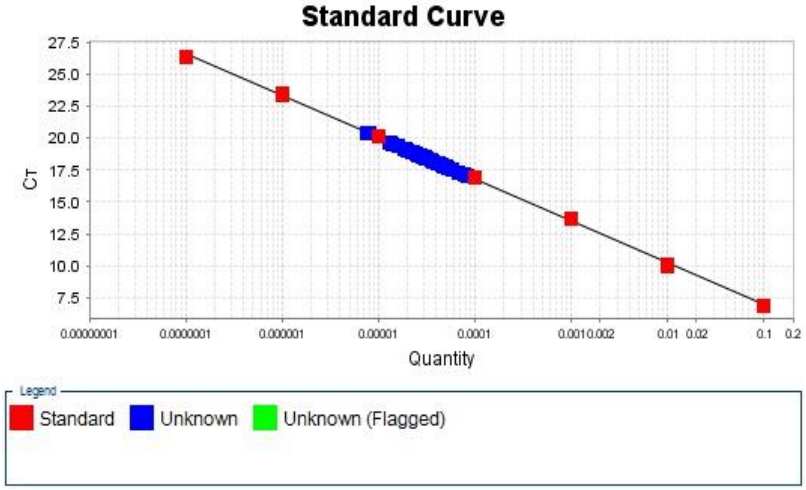



**Figure 2.12 Representative standard curve from qPCR.**

A linear graph of the exponential PCR is formed by plotting the cycle number at Ct against the known concentrations of DNA in ten-fold dilution series, represented logarithmically. Correlation coefficient ($R^2$) and reaction efficiency (E) are then calculated.

### 2.5.4.3 Quantification of gene expression

Before quantifying gene expression, it is important to normalise for the variation in initial RNA abundance. This can be done by comparing the expression of the gene of interest against the reference gene, which is a gene that is expressed at a relatively constant high level in all cells. Using this method, a relative quantification of gene expression can be calculated using a mathematical model known as the comparative

threshold ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen 2001), where the relative quantities of the target gene are divided by the geometric mean of the most stable reference genes (Section 2.5.6). The relative expression of target genes was determined using the geNorm method (geNorm software v3.5; Primer Design, Ltd; Southampton, UK) taking into account the individual efficiency of each experiment with an acceptable efficiency range falling between 90 and 105% (Pfaffl, 2001; Arya et al. 2005).

### 2.5.5 Selection of the Reference Gene in qPCR

The reference gene(s) is a cellular RNA that is amplified with the target and commonly used as an internal control in RNA quantification and against which the target RNA values can be normalised. However, these reference genes vary greatly between samples, so using the wrong reference gene could lead to incorrect results (Tricarico et al., 2002). It is essential to select gene(s) that are widely and stably expressed in the particular tissue samples being examined (Dorak, 2007) such that their expression level is relatively higher than that of any of the genes of interest (Silver, 2006).

Genes such as IPO8, TBP, RPLP0 and KDM2B have been commonly used as reference genes in previous studies (Hurtado Del Pozo et al., 2010; Bonnet et al., 2013). In theory, all of these genes have a stable level of expression across all samples cells, though, practically, none of them is ideal in terms of stable levels of expression and low variation between samples. Therefore, all four previously mentioned genes were analysed for each set of samples and the crossing threshold values for each reference gene processed through the geNorm software v3.5 (Primer Design, Ltd; Southampton, UK). The software compares the geometric mean expression and pairs the variation of each selected gene with all other genes in order to calculate a stability value (M) for each. It then selects the two genes with the lowest M value as the normalisation genes, as those with the lowest M values have the most stable expression levels (Vandesompele et al., 2002).

### 2.5.6 Development of Primers for qPCR

Primer sequences used in this study were either taken from published studies or designed in-house using the sheep genome sequence from the National Center for Biotechnology Information (NCBI) online database (http://www.ncbi.nih.gov). Using this database, primers were designed to include an exon-exon junction, where possible, with one primer flanking the exon-intron boundary. In order to achieve the most favourable reaction conditions with greater amplification specificity, care was taken to select primers that have optimum annealing temperatures, guanine-cytosine base pair content and length. Primers designed in-house were sequenced and the results were checked against known sequences on the GenBank Database. An additional comparison against the bovine/ovine genome was done for all primers using the NCBI nucleotide Basic Local Alignment Search Tool (BLAST) database (http://www.ncbi.nlm.nih.gov/BLAST) to ensure the correct selection of sequences. Primers were developed by and acquired from Sigma Aldrich (Sigma-Aldrich Company Ltd.; Dorset, UK). Primers were stored in nuclease-free water at a stock concentration of 100 µmol/l. All primer sequences used in mRNA quantification are detailed in Tables 2.14 and 2.15.

**Table 2.14 Reference gene primer sequences for mRNA quantification.**

| Reference Gene | Reference or NCBI number | Primer sequence (5'-3') |
|---|---|---|
| IPO8 | Pope et al. (2014) | F GCCCTTGCTCTTCAGTCATT<br>R GTGCAACAGCTCCTGCATAA |
| RPLP0 | Robinson et al. (2007) | F CAACCCTGAAGTGCTTGACAT<br>R AGGCAGATGGATCAGCCA |
| KDM2B | XM_015101402.1 | F GGGTGGGAAGATCTTTTGGCT<br>R TCCAGCCGGAAGGGATGAAAA |
| TBP | XM_015097549.1 | F CTTGGACTTCAAGATTCAGAACA<br>R CCAGGAAACTCTGGCTCA |

**Table 2.15 Primer Sequences for mRNA Quantification of Genes of Interest.**

| Gene | Reference or NCBI number | Primer sequence (5'-3') |
|---|---|---|
| ACOT11 | XM_015092003.1 | F CCCTCGTCTGCGCAAGA<br>R TGTATCCACAAGCCATTTAATGCT |
| COX1 | NM_001009476.1 | F CAGTGCGTTCCAACCTTATCC<br>R GGACTCCCAGCTGATGTAATCG |
| FAS | XM_015098375.1 | F ATGATGACAGGCGGTGGAA<br>R ATACCGGGACAGGTCCTCAG |
| SCD1 | NM_001009254.1 | F GGAGTCACCGAACCTACAAAGC<br>R TGGAACGCCATGGTGTTG |
| COX2 | NM_001009432.1 | F CCATGCCAGAATCGAGGTGT<br>R GTGTCGGGAGTGGGTTTCAG |
| FADS2 | XM_015103138.1 | F CGCTGGGAGGAGATTCAGAA<br>R ATGTTGTAGACCTTGCGATCGA |
| FABP3 | NM_001267884.2 | F CCTGGAAGTTAGTGGACAGCA<br>R TGATTGTGGTAGGCTTGGTCA |
| UCP1 | Budge et al. (2004) | F AGAGGTGGTCAAGGTCAG<br>R ATTCTGTAAGCATTGTAAGTCC |
| PRDM16 | XM_003583245.1 | F TGGCAGCTGGCTCAAGTACA<br>R CGGAACGTGGGCTCCTCATC |
| Leptin | Pope et al. (2014) | F CCAGGATGACACCAAAACC<br>R TGGACAAACTCAGGAGAGG |
| HOXC9 | XM_002704244.2 | F GACCTGGACCCCAGCAAC<br>R GCTCGGTGAGGTTGAGAAC |
| SHOX2 | NM_001205527.1 | F CGCCTTTATGCGTGAAGAAC<br>R TTGGCTGGCAGCTCCTAT |
| PPARγ | Pope et al. (2014) | F GACCCGATGGCTCCGTTA<br>R TGAGGGAGTTGGAAGGCTCT |
| RIP140 | Pope et al. (2014) | F CGAGGACTTGAAACCAGAGC<br>R TCTTAGGGACCATGCAAAGG |

### 2.5.7 Classical Polymerase Chain Reaction Procedure

Classical PCR was performed in order to optimise the primer conditions and create cDNA qPCR standards. Eppendorf tubes (0.2 ml) were labelled for each sample and for the NTC. The Master Mix was prepared such that it contained the correct quantities of Thermo-Start® PCR Master Mix (ABgene LTD., UK) and the forward and reverse primers were diluted 1:40 (unless otherwise stated) for each sample using the volumes given in Table 2.16.

**Table 2.16 Master Mix reagents and quantities used for polymerase chain reaction (PCR) samples.**

| Reagent | Volume (µl ) |
|---|---|
| Stock Thermo-Start PCR Master Mix | 10 |
| Forward primer (1:40 dilution) | 1 |
| Reverse primer (1:40 dilution) | 1 |
| Nuclease-free water | 7 |

Volumes were multiplied by the number of samples, including the NTC, and an additional 4 % of each reagent was added to allow for pipetting errors and pipette tip waste.

Then, 19 µl of the Master Mix was added to each Eppendorf tube along with 1 µl of the corresponding cDNA sample, using nuclease free water in the case of the NTC. One negative control, with no template cDNA or primers, was run at the same time to ensure the integrity of the RT-PCR reaction and as a quality control for gDNA contamination. All samples were centrifuged for 1 minute at room temperature at 10,000 g and then loaded into the PCR machine (TouchGene Gradient; Techne; Cambridge, UK) with a pre-set program (Table 2.17).

**Table 2.17 Classical polymerase chain reaction (PCR) standard program details. The second through fifth steps of the program repeat for 40 cycles.**

| Process | | Temperature (°C) | Time |
|---|---|---|---|
| Initiation and enzyme activation | | 94 | 30 seconds |
| 40 cycles | Denaturing cDNA strands | 94 | 30 seconds |
| | Primer annealing | 60 | 30 seconds |
| | Primer extension | 68 | 1 minute |
| Final extension | | 68 | 5 minutes |
| Hold | | 8 | ∞ |

## 2.5.8 Agarose Gel Electrophoresis and DNA Extraction

DNA samples are run on an agarose gel and then analysed in order to confirm the presence of the gene of interest in the sample. Moreover, by using a negative control, any contamination in the samples can be determined through the presences of multiple bands or a band in the negative control lane. The negative control also detects nonspecific amplification, which occurs when complementary bases within the primers attach to each other to create primer dimers, which are represented by more than one band in the gel matrix.

DNA fragments can be separated according to their size by applying an electric field to DNA in an agarose gel matrix, which causes the DNA fragments migrate towards the positive electrode due to their negative charge. Because of the presence of the agarose matrix, smaller fragments move further and faster (Lodish, 2008). To visualise the DNA, ethidium bromide is added to the separating medium. Due to its fluorescent characteristics when exposed to UV light, it allows for the detection of the DNA fragment bands within the matrix. A DNA ladder composed of DNA fragments of known sizes is run alongside the samples in order to identify band sizes.

### 2.5.8.1 Agarose Gel Electrophoresis Procedure

A 2% (w/v) agarose gel was prepared by dissolving 1 g of agarose powder (Invitrogen Life Technologies) in 50 ml TAE buffer (made from a stock solution of tris

(hydroxymethy) aminomethane base (Tris), glacial acetic acid (Fischer Scientific) and EDTA buffer). The solution was heated to dissolve the gel and slightly cooled before 2.5 µl of a 10 mg/ml solution of ethidium bromide was added. The gel was then poured into a cassette and combs were inserted to create wells that would hold the DNA. After the gel was formed, the combs were carefully removed and the gel was placed in an electrophoresis tank filled with enough 1x TAE buffer to cover the gel.

The samples and DNA ladder were loaded onto the gel. The first well contained 5 µl of a 100 base pair (bp) DNA ladder (Fisher Scientific, Loughborough, Leicestershire, UK) followed by the amplified samples in the following wells. After loading, the samples were passed through the gel at 110 volts for 40 minutes, or until the dye had proceeded to a suitable distance to ensure separation. On completion of the run, the gel was visualised and imaged using a UV trans-illuminator CCD camera (Fujifilm luminescent image analyser LAS-3000 v1.01; Raytek; Sheffield, UK). Fluorescent gel bands were cut out of the gel and placed into a labelled, sterile, 1.5 ml Eppendorf tube and stored at -20°C until the DNA extraction was performed.

#### 2.5.8.2 DNA Extraction Procedure

The pre-cut DNA bands were extracted from the gels using a QIAquick gel extraction kit (Qiagen; West Sussex, UK). Gel bands were first weighed and dissolved in 300 µl buffer QG (included in the kit) per 100 µg of gel by heating for 10 minutes at 50°C with vortexing every 2–3 minutes. Following this, 100 µl of isopropanol (Fisher Scientific, Loughborough, Leicestershire, UK) per 100 µg of gel was added and mixed by vortex. The samples were then pipetted into a QIAquick spin column placed in a 2 ml collection tube (included in the kit) and centrifuged at 10000 g for 1 minute at room temperature, discarding any flow-through. Next, 500 µl of buffer QG was added to the columns and they were centrifuged for 1 minute at 10000 g at room temperature to remove residual agarose, and again the flow-through was discarded. The columns were washed with 750 µl of buffer PE (premade buffer, included in the kit), incubated for 3 minutes, and then centrifuged for 1 minute at 10000 g at room temperature. The columns were then centrifuged again for an additional 1 minute at

10000 g at room temperature to ensure the removal of all remaining PE buffer. The columns were then transferred to a new, sterile, 1.5 ml Eppendorf tube and 50 µl of premade elution buffer (Buffer EB, 10mM Tris·Cl, pH 8.5, included in the kit) was added directly onto the QIAquick membrane and centrifuged for 1 minute at 10000 g at room temperature.

DNA concentration was measured using a Nanodrop® spectrophotometer (Nanodrop Technologies; Wilmington, USA). For newly designed primers, DNA samples were sequenced at the University of Nottingham's Centre for Genetics and Genomics (Queen's Medical Centre; Nottingham, UK) and referenced against the NCBI online database. Samples were diluted to 1 ng/µl and stored at -20°C until needed.

### 2.5.9 Quantitative PCR Procedure

In order to calculate the efficiency of the PCR reaction, a standard curve was used. For each gene, a known concentration of cDNA samples extracted from the agarose gels was used to create a standard curve. A 1:10 serial dilution series with concentrations ranging from 0.1 to 1 x $10^{-8}$ ng/µl was prepared in Eppendorf tubes. Samples were loaded in duplicate with a negative control (nuclease-free water) and positive control (random cDNA sample) to verify the efficiency of the experiment

A master mix containing SYBR Green Master Mix, a forward primer and a reverse primer was prepared, and 7 µl of this mix was loaded into each well of a sterile 96 well PCR plate (Abgene; Thermo Fisher Scientific Inc.; UK). Following this, 3 µl of the cDNA samples, the known concentration standards (standard curve) or nuclease free water (negative control) were loaded into the corresponding wells. Samples and standards were run in duplicate to ensure the efficiency of the experiment. Negative controls missing either the template cDNA or the primers and positive controls for the same cDNA samples were run in all plates for each gene analysed to allow for data calibration. Quantities of samples and reagents are listed in Table 2.18.

**Table 2.18 Reagents and quantities used for quantitative polymerase chain reaction (qPCR) samples.**

| Reagent | Per sample (µl) |
|---|---|
| SYBR Green Master Mix | 5 |
| 1:40 dilution of forward primer | 1 |
| 1:40 dilution of reverse primer | 1 |
| **Subtotal for Master Mix** | **7** |
| cDNA (for samples)<br>known concentration standards (for standards curve)<br>nuclease free water (for negative controls) | 3 |
| **Total volume** | 10 |

Once prepared, the plate was sealed using an Abgene plate sealer and thermal seals (Alpha Laboratories; Hampshire, UK), placed in the StepOne Plus Real Time PCR System (Applied Biosystems; Warrington, UK) and the qPCR was run on the pre-set program detailed in Table 2.19.

**Table 2.19 Details of the qPCR standard program.**

| Process | | Temperature | Time |
|---|---|---|---|
| Initial denaturing of cDNA strand | | 95°C | 15 minutes |
| 45 cycles | Denaturing cDNA strand | 95°C | 15 seconds |
| | Annealing of primers | 58-62°C | 30 seconds |
| 31 cycles | Melt curve analysis | 65 to 95°C in 1°C increments | 30 seconds |
| Hold | | 95°C | 10 minutes |

### 2.5.10 Microarray analysis

A microarray is a laboratory appliance used to detect and then arrange the expression of thousands of identified sequenced genes at the same time by using an impermeable solid support glass or silicon chip. It investigates the similarities and differences in the gene expression patterns in the target samples, which, in turn, enrich current knowledge on the mechanisms involved in biological processes. Each sequenced gene matches a fragment of gDNA, cDNA, PCR products or chemically synthesised oligonucleotides that represent a single gene, which is then used to run a technology through which parallel gene expression analyses of thousands of known and unknown gene functions are conducted .

To create the microarray target, the extracted mRNA and the reference are converted into cDNA with a reverse-transcriptase enzyme in the presence of a short primer so that cDNA synthesis is initiated. Then, each cDNA is labelled with a different tracking molecule, usually fluorescent cyanine dyes, before the product is washed off to remove any contaminants, such as proteins, extra primers and unincorporated nucleotides.

The microarray slides are then incubated at a high temperature with a solution containing saline-sodium buffer, sodium dodecyl sulphate and bovine serum albumin to minimise non-specific binding. The labelled cDNA is competitively hybridised against the oligonucleotides on the immobilised array, and binds only to its probe sequence on the microarray chip. The slides are then washed to remove any non-hybridised cDNA fragments, dried and placed in a scanner to quantify the labelled cDNA that bound to each target spot (feature). The strength of the signal of each spot, which depends on the amount of targets that bound to the probes present on that spot, is measured with a fluorescent microscope. Relative quantitation, in which the intensity of a feature is compared with the intensity of the same feature under a different condition, is analysed, and the identity of the feature is known by its position.

#### 2.5.10.1 Microarray analysis procedure

The microarray analysis was performed at Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, School of Biosciences. mRNA extracted from sternal, prerinal and epicardial adipose tissue samples was tested for integrity with the Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, USA), and subsequent mRNA processing was completed with the GeneChip® 3' IVT Express kit (Affymetrix, High Wycombe, UK). A previous study had demonstrate that the Affymetrix Human U133+2 gene chip array can be used to study gene expression profiles in sheep tissues (Graham et al., 2011). All protocols were performed according to the manufacturer's instructions. Each GeneChip® was loaded into Affymetrix® Genechip® Scanner 3000 7G (Affymetrix, High Wycombe, UK) for fluorescence analysis. Quality control of labelling and hybridisation, as well as data analysis, was conducted with Partek (Partek Inc., St. Louis, USA). Data normalisation and analysis were done with the assistance of Dr Hernan Fainberg and Mr Ahmad Alhaddad (Department of Academic Child Health, University of Nottingham).

## 2.6 Statistical Analysis

In general, statistical analysis was conducted and graphs were created with the software package GraphPad Prism v6.0 (GraphPad Software, California, USA). Data are presented as mean ± standard error of the mean (SEM).

All data were subjected to the Kolmogorov-Smirnov normality test for parametric or non-parametric distribution, where $p \geq 0.05$ determined normality. Parametric tests were used when the data are small sets, thus, unsuitable for non-parametric tests (Bland & Altman, 2009).

When the aim of studies was to examine the effect of supplementation, one-way ANOVA was used to test the significance of the control versus canola and/or the control versus sunflower because a direct comparison of the supplementation results provided no benefit. However, when the underlying purpose of the study was to

compare the results of interest among the examined adipose tissue depots, parametric data were assessed with oneway ANOVA, followed by multiple group post hoc correction tests.

Statistical analysis for the initial quality control of labelling and hybridisation, as well as data analysis of the gene expression and microarray results was conducted with Partek software (Partek Inc., St. Louis, USA).

Normalization and network analyses were performed, unless otherwise stated, using free and open source packages from the R project (http://cran.r-project.org/). Data were evaluated using the "Limma" library, which allowed performing empirical Bayesian statistical modelling between milk samples as well as the three adipose tissue depots and the effect of the maternal fatty acid supplementation. The R-package "gplots" was used to assess fold changes, and make heat maps, and expression plots.

Any additional statistical tests used will be detailed in the relevant chapters. The results were considered significant when $p \leq 0.05$.

No power calculations were performed before the investigations, but where necessary, false discovery rate (FDR) approach was conducted and considered $q \leq 0.05$ as significant (Wettenhall & Smyth, 2004.).

# Chapter 3. The effects of manipulating dietary fatty acid supplementation on milk fatty acid profiles

## 3.1 Introduction

### 3.1.1 Overview

Two outcomes were measured in this study and are summarised in Figure 3.3. The first, tested the effects of supplementing the maternal diet with canola or sunflower oil on milk fatty acid (FA) profiles in sheep. The second, covered in Chapter 4, investigated the effects of these changes in the milk FA profile on the body composition of offspring, plasma metabolites and the thermogenic capacity of major brown adipose tissues.

### 3.1.2 Fatty acids

FAs consist of a carboxyl group with a long hydrocarbon chain and can be either saturated or unsaturated. When all the carbon-carbon bonds are single the FA is classified as 'saturated'; when one or more of the bonds is a double or triple bond, the FA is classified as 'unsaturated'. FAs that contain one double bond are known as monounsaturated fatty acids (MUFAs), whilst those with more than one double bond are known as polyunsaturated fatty acids (PUFAs). These, in turn, can be classified into various groups depending on their chemical structure. Notably, omega (ω) FAs are classes of PUFAs which are essential for human health.

The position of a double bond in the carbon atom at the end of the carbon chain determines FAs' omega class, which can be either 3, 6 or 9. Mammals lack the necessary desaturase enzymes to create double bonds closer than the ninth carbon atom (Berg et al., 2002; Bradbury, 2011; Nakamura & Nara, 2004); thus, ω-3 and ω-6 FAs must be obtained from their diet, and are therefore named 'essential' FAs. Excluded from this are the more complex ω-3 and ω-6 FAs: α-linolenic acid (ALA; 18:3 n-3) and linoleic acid (LA; 18:2 n-6) (Figure 3.1), both of which can be synthesised in

the body by a process of elongation and desaturation from these precursors (Barceló-Coblijn & Murphy, 2009) that are mainly obtained from vegetables, vegetable oils and cereals (Meyer et al., 2003).

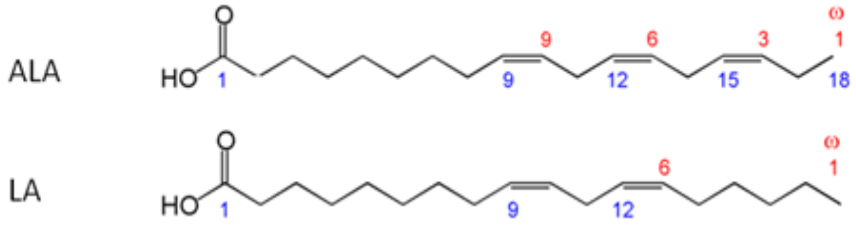



**Figure 3.1 Structural representation of ALA (ω-3) and LA (ω-6).**
Numbers in red represent the carbon atoms counted from the methyl end of the chain. Numbers in blue represent the carbon atoms counted from the carboxyl end.

The ω-3 and ω-6 fatty acids provide many benefits to human health. Those of ω-3 are attributed to its anti-inflammatory effects, cardiovascular disease prevention properties and involvement in neuronal cell development (Klek, 2016; Swanson et al., 2012). The ω-6 acid is beneficial in immune response, which derives many bioactive signalling molecules from the acid (Mittal et al., 2010). The diet should be balanced in an ω-6: ω-3 ratio, as they both compete for the same desaturase and elongase enzymes (Barceló-Coblijn & Murphy, 2009; Lorente-Cebrián et al., 2013; Simopoulos, 2002). However, in modern Western diets, there is a tendency to over-supply ω-6 and under-supply ω-3, with ratios ranging between 15:1 to 17:1 (Ailhaud et al., 2006; Simopoulos, 2002). Thus, this imbalance may favour the conversion of LA to arachidonic acid (AA) over the conversion of ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ailhaud et al., 2006), which in turn can promote the development of many chronic diseases (Simopoulos, 2002) (Figure 3.2).

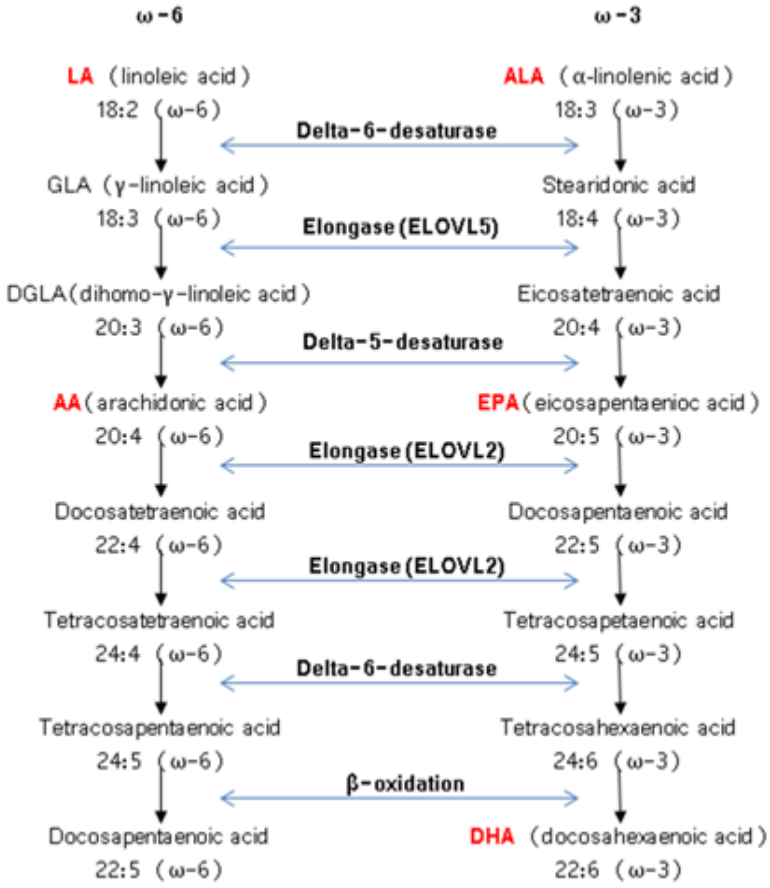



**Figure 3.2 Metabolism of ω-3 and ω-6 fatty acids in the human body.**

LA (ω-6) and ALA (ω-3) are further processed by a series of reactions catalysed to become longer carbon chains with more double bonds by the same set of enzymes: desaturase and elongase. β-oxidation is the final step. Figure adapted from Russo, 2009.

### 3.1.3 Supplements used for this investigation

There is growing evidence for the association between early life nutrition and health outcomes in the long term, with early-onset childhood obesity being linked to obesity later in life (Agostoni et al., 2013; Young et al., 2012). In the early months of life, milk is the only food consumed, with breast milk considered the optimal form of nutrition (Jeurink et al., 2013). Although the relationship between early-life feeding and later risk of childhood obesity is complex and confounding (Berti et al., 2017; Farrow et al., 2013), breastfeeding likely has a protective effect against future obesity (Baird et al., 2008; Hörnell et al., 2013; Moorcroft et al., 2011).

As discussed in Chapter 1, brown adipose tissue controls energy balance by regulating energy metabolism through thermogenesis, which is mediated by the unique action of UCP1 (Himms-Hagen, 1990). The functions of adipose tissues, white and brown, could be affected by dietary factors, such as dietary fat type (Takahashi & Idl, 1999). In humans, a number of studies have shown that ω-3 FAs have fat-lowering effects (Buckley & Howe, 2010; Flachs et al., 2009). In rats, dietary fat with a high content of ω-6 (Mercer & Trayhurn, 1987; Shillabeer & Lau, 1994) and ω-3 (Belzung et al., 1993; Oudart et al., 1979) PUFAs cause less body fat accumulation compared to saturated fatty acids (SFAs).

It is possible to alter milk FA profiles by modifying the diet (Innis, 2007). As mentioned in Chapter 1, numerous studies have used dietary FAs to improve ruminants' milk composition for the benefit of human health (Dewhurst et al., 2006; Lock & Bauman, 2004; Shingfield et al., 2006). In this investigation, canola and sunflower oil were used to alter the FA profiles of ewes' milk due to their ability to increase MUFAs and decrease SFAs in milk (Hervás et al., 2008; Okine et al., 2003; Toral et al., 2010; Welter et al., 2016).

In the present study, maternal diet was supplemented with 3% canola oil or 3% sunflower oil based on other studies that found an alteration in milk FA profiles, but not milk fat content, with similar doses ranging from 2% to 4% (Mir et al., 1999; Okine et al., 2003). The components of the diets are shown in Table 3.1.

**Table 3.1 The dietary information for the control and intervention groups (Manor Farm Feeds (Owston) Ltd, Leicestershire, UK).**

| Constituent | Control (%) | Canola (%) | Sunflower (%) |
|---|---|---|---|
| Canola oil | 0 | 3 | 0 |
| Sunflower oil | 0 | 0 | 3 |
| Other crude oils and fats | 3 | | |
| Crude protein | 18 | | |
| Crude fibre | 7 | | |
| Crude ash | 6 | | |
| Moisture | 15 | | |
| Calcium | 0.5 | | |
| Sodium | 0.2 | | |
| Phosphorus | 0.1 | | |
| Magnesium | 0.1 | | |
| Additives | Per Kg | | |
| Vitamin A {E627} | 10000 iu | | |
| Vitamin E (all-rac-alpha-tocopheryl acetate) {3a700} | 150 mg | | |
| Vitamin D3 {E671} | 2000 iu | | |
| Manganese {E5} Manganous oxide | 129 mg | | |
| Zinc {E6} Zinc oxide | 111 mg | | |
| Iron {E1} Ferrous Sulphate, monohydrate | 97 mg | | |
| Iodine {E2} Calcium Iodate, anhydrous | 7.9 mg | | |
| Cobalt {E3} Basic cobaltous carbonate, monohydrate | 4 mg | | |
| Selenium {E8} Sodium Selenite | 1.1 mg | | |

### 3.1.3.1 Fatty acid composition of supplements

Canola and sunflower oil were selected for this investigation due to their high unsaturated fatty acid (UFA) content (c. 82% and 83%, respectively). According to the British Nutrition Foundation (2006), 'It is becoming increasingly clear that UFAs have important health effect'. The FA compositions of the oil supplements utilised in this study are shown in Table 3.2. Regardless of their compositional differences, canola oil has higher proportion of PUFAs and a relatively lower proportion of MUFAs (c. 65% and 26%, respectively) than the sunflower oil (c. 46% and 37%, respectively). With regard to omega FAs, canola oil is higher in ω-6 and lower in ω-9 compared with sunflower oil, and both are low in ω-3.

**Table 3.2 Fatty acid composition of canola and sunflower oils.**

| Fatty acids * | | Canola (%) | Sunflower (%) |
|---|---|---|---|
| **Saturated** | C 10:0 | 0.07 | 0.03 |
| | C 12:0 | 0.66 | 0.03 |
| | C 14:0 | 0.38 | 0.16 |
| | C 15:0 | 0.07 | 0.10 |
| | C 16:0 | 12.94 | 13.59 |
| | C 17:0 | 0.08 | 0.10 |
| | C 18:0 | 2.77 | 1.69 |
| | C 20:0 | 0.29 | 0.46 |
| | C 22:0 | 0.68 | 0.48 |
| | C 23:0 | 0.00 | 0.00 |
| | C 24:0 | 0.29 | 0.27 |
| | **Total SFAs** | **18.23** | **16.91** |
| **Unsaturated** | C16:1 n-7 | 0.17 | 0.22 |
| | C18:1 n-9 | 23.82 | 33.42 |
| | C18:1 n-7 | 0.85 | 1.69 |
| | C20:1 n-9 | 0.37 | 0.84 |
| | C20:3 n-9 | 0.04 | 0.02 |
| | C22:1 n-9 | 0.06 | 0.18 |
| | C24:1 n-9 | 0.19 | 0.27 |
| | C18:3 n-3 | 2.45 | 5.76 |
| | C22:3 n-3 | 0.24 | 0.20 |
| | C18:2 n-6 | 53.01 | 39.77 |
| | C18:3 n-6 | 0.01 | 0.03 |
| | C20:2 n-6 | 0.17 | 0.27 |
| | C20:3 n-6 | 0.05 | 0.04 |
| | C20:4 n-6 | 0.04 | 0.06 |
| | C24:2 n-6 | 0.11 | 0.16 |
| | **Total MUFAs** | **25.50** | **36.64** |
| | **Total PUFAs** | **56.08** | **46.28** |
| | **Total UFAs** | **81.58** | **82.92** |

SFAs are saturated fatty acids; MUFAs are monounsaturated fatty acids; PUFAs are polyunsaturated fatty acids; UFAs are unsaturated fatty acids.

* C10:0 = capric; C16:0 = lauric; C14:0 = miristic; C15:0 = pentadecylic; C16:0 = palmitic; C17:0 = margaric; C18:0 = stearic; C20:0 = arachidic; C22:0 = behenic; C23:0 = tricosylic; C24:0 = lignoceric; C16:1 = palmitoleic; C18:1 n-9= oleic; C18:1 n-7= vaccenic; C20:1 = gadoleic; C20:3 = mead; C22:1 = erucic; C24:1 = nervonic; C18:3 = linolenic; C22:3 = docosatrienoic; C18:2 = linoleic; C18:3 = γ-linolenic; C20:2 = eicosadienoic; C20:3 = dihomo- γ-linolenic; C20:4 = arachidonic; C24:2 = lignoceric.

## 3.2 Hypotheses

I hypothesise that supplementing maternal diet during lactation with 3% canola oil or 3% sunflower oil affects milk FA profiles by:

- decreasing SFAs.
- increasing MUFAs.
- increasing total *trans* fat.

## 3.3 Methods

The experimental model used in this chapter is summarised in Figure 3.3, while the methods are described in detail in Chapter 2. All animal experimentations for this study were performed by Professor Michael Symonds, Dr. Mark Birtwistle, Dr. Viv Perry and Dr. Graeme Davies at Sutton Bonington campus, University of Nottingham. Milk samples were analysed at The University of Pecs, Hungary, by Dr. Tamás Marosvölgyi.

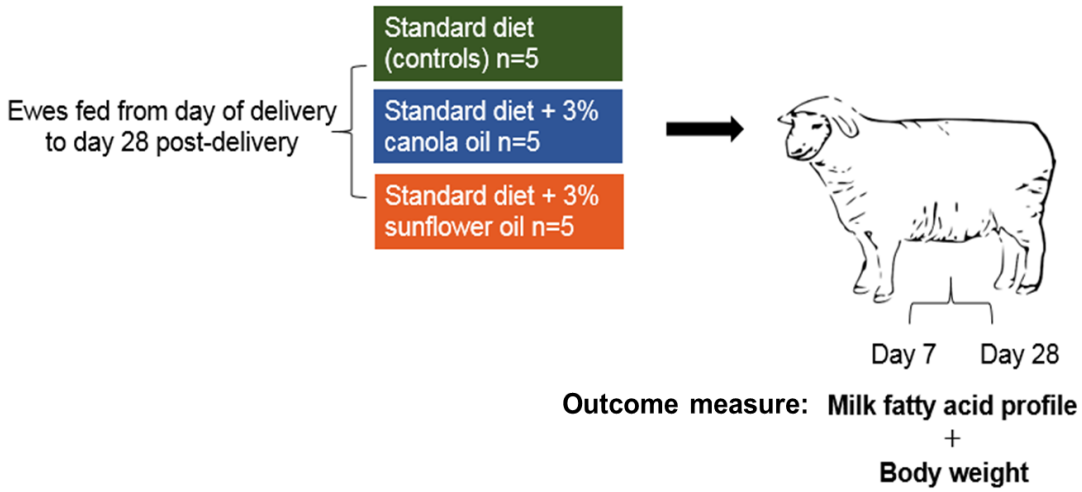



**Figure 3.3 Summary of experimental model used in Chapter 3.**
Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil. Offspring lambs were mother-fed from birth until day of tissue sampling. Day numbers refer to days of age. At days 7 and 28, ewes underwent body weight and milk samples were collected.

Milk analysis was not possible for all the ewes in this study because of difficulty obtaining milk samples or because a complete analysis could not be completed due to either contamination or insufficient sample quantity. In the control group, milk could not be obtained from two ewes at day 7 and one ewe at day 28. In the canola group, it was not possible to collect milk from three ewes at day 7 and one ewe at day 28. In the sunflower group, no milk samples were obtained from three ewes at day 7 and two ewes at day 28. Milk fat analyses, including total fat, SFAs, MUFAs, ω-3, ω-6 and ω-9 FAs, and *trans* fatty acids (TFAs), were performed. However, due to one or more

of the reasons mentioned above, analysis was not completed for a further seven ewes at day 7 (three in the control group, one in the canola group, and three in the sunflower group) and four at day 28 (two in the canola group and two in the sunflower group). Therefore, the final numbers of milk samples obtained were as follows: at day 7, five in the control group, five in the canola group, and three in the sunflower group; at day 28, eight in the control group, six in the canola group, and five in the sunflower group. Numbers of milk samples are summarised in Table 3.3.

**Table 3.3 Summary of milk samples numbers in Chapter 3.**

| | | Control (*n*=10) | Canola (*n*=9) | Sunflower (*n*=9) |
|---|---|---|---|---|
| **Day 7** | S | 8 | 6 | 6 |
| | NS | 2 | 3 | 3 |
| | NA | 3 | 1 | 3 |
| | **Total** | **5** | **5** | **3** |
| **Day 28** | S | 9 | 8 | 7 |
| | NS | 1 | 1 | 2 |
| | NA | 0 | 2 | 2 |
| | **Total** | **8** | **6** | **5** |

S is sampled; NS is non-sampled; NA is not analysed due to either contamination or insufficient sample quantity.

In order to minimise potential variability in milk FA profiles as a result of hormonal differences (Bauman & Elliot, 1983; Tucker, 1985), feeding patterns (Linn & Otterby, 1984) and some within-breed genetic variation (Linn, 1988), the analysis focused on milk samples extracted from ewes on both day 7 and day 28. This resulted in five samples per group at each time point (except in the sunflower group at day 7).

### 3.3.1 Statistical analysis

Normalisation and data analysis were performed using free and open source packages from the R project (http://cran.r-project.org/). The 'gcrma' function embedded in the 'affy' package was used for pre-processing data stages, including background correction, normalisation and probe match verification. Results are expressed as % milk fat. For the statistical analysis, in order to compare between the milk samples in

the control and two nutritional intervention groups, one-way ANOVA was used to test the significance of the control versus canola and the control versus sunflower oil supplemented groups and the false discovery rate (FDR) approach was applied. The results were considered significant when $p \leq 0.05$. R-package 'gplots' were used to create the heat map.

## 3.4 Results

### 3.4.1 The effects of supplementing maternal diet with fatty acids on milk fatty acid profiles in the first month of lactation in sheep

#### 3.4.1.1 Total fat

Supplementing maternal diet with canola oil or sunflower oil had no effect on total milk fat content at either day 7 or day 28, as shown in Figure 3.4.

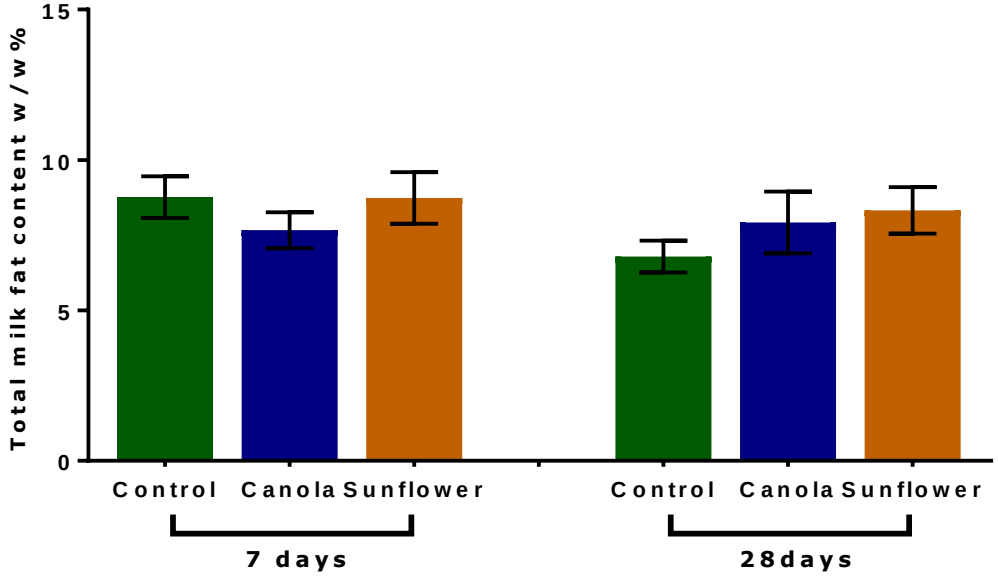



**Figure 3.4 Milk fat content (w/w%: percentage by weight of total solution) at days 7 and 28 of lactation.**

Milk samples were taken from ewes fed a standard diet (Control: day 7, *n* = 5; day 28, *n* = 5), a standard diet + 3% canola oil (Canola: day 7, *n* = 5; day 28, *n* = 5) and a standard diet + 3% sunflower oil (Sunflower: day 7, *n* = 3; day 28, *n* = 5). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. There were no significant differences ($q \geq 0.05$).

#### 3.4.1.2 Saturated fatty acids

Maternal supplementation with canola oil or sunflower oil did not have any significant effects on short-chain (< 6 carbons long) FA concentrations in milk at either day 7 or day 28 (Tables 3.3 and 3.4).

All medium-chain FAs (6–12 carbons long) – caprylic acid (C8:0), capric acid (C10:0), undecylic acid (c11:0) and lauric acid (C12:0) – were decreased by 20–30% (except undecylic acid, with a decrease of about 70%) in the canola group's milk compared to the control by day 7 of lactation; the same result did not occur in the sunflower group. By 28 days of lactation, the effect of maternal supplements was limited to a reduction of C10:0 and C12:0 medium-chain FAs in the milk of the canola group, but did lead to a reduction of C8:0, C10:0 and C12:0 content in the milk of the sunflower group (Tables 3.4 and 3.5).

Concentrations of long-chain FAs (LCFAs) (> 12 carbons long) – myristic acid (C14:0) and palmitic acid (C16:0) – decreased in the milk of the canola group at day 7 of lactation, but not in the milk of the sunflower group; at day 28, however, concentrations decreased to a similar extent in the milk of both the canola and sunflower groups. In contrast, stearic acid (C18:0) and arachidic acid (C20:0) were increased in the milk of the canola group, but not in the milk of the sunflower group, at day 7 of lactation; and, by day 28, C18:0 was increased only in the milk of the canola group, while C20:0 was increased in the milk of both the canola and sunflower groups. Finally, concentrations of behenic acid (C22:0) increased by 50% in the milk of the sunflower group, but only at day 28 of lactation (Tables 3.4 and 3.5).

**Table 3.4 Saturated fatty acid content (% of fat) of milk at day 7 of lactation.**

| Fatty acid | Common name | Control ($n$ = 5) | Canola ($n$ = 5) | Effect (% change) | Sunflower ($n$ = 3) | Effect (% change) |
|---|---|---|---|---|---|---|
| **C4:0** | **Butyric Acid** | 2.55 ± 0.06 | 2.61 ± 0.07 | NS | 2.77 ± 0.11 | NS |
| **C6:0** | **Caproic Acid** | 2.11 ± 0.04 | 1.71 ± 0.12 | 23%↓ $q$ = 0.04 | 1.93 ± 0.11 | NS |
| **C8:0** | **Caprylic Acid** | 1.76 ± 0.08 | 1.27 ± 0.14 | 28%↓$q$ = 0.04 | 1.52 ± 0.18 | NS |
| **C10:0** | **Capric Acid** | 2.56 ± 0.10 | 1.87 ± 0.20 | 27%↓ $q$ = 0.04 | 2.13 ± 0.29 | NS |
| **C11:0** | **Undecylic Acid** | 0.07 ± 0.01 | 0.02 ± 0.01 | 71%↓ $q$ = 0.04 | 0.05 ± 0.03 | NS |
| **C12:0** | **Lauric Acid** | 1.95 ± 0.08 | 1.49 ± 0.11 | 24%↓ $q$ = 0.04 | 1.56 ± 0.16 | NS |
| **C13:0** | **Tridecylic Acid** | 0.08 ± 0.01 | 0.04 ± 0.01 | NS | 0.06 ± 0.01 | NS |
| **C14:0** | **Myristic Acid** | 3.00 ± 0.06 | 2.63 ± 0.09 | 12%↓ $q$ = 0.04 | 2.56 ± 0.10 | NS |
| **C15:0** | **Pentadecylic Acid** | 0.68 ± 0.04 | 0.55 ± 0.05 | NS | 0.57 ± 0.03 | NS |
| **C16:0** | **Palmitic Acid** | 4.50 ± 0.03 | 4.34 ± 0.02 | 4%↓ $q$ = 0.03 | 4.31 ± 0.03 | NS |
| **C17:0** | **Margaric Acid** | 0.93 ± 0.03 | 1.01 ± 0.04 | NS | 0.98 ± 0.005 | NS |
| **C18:0** | **Stearic Acid** | 3.84 ± 0.08 | 4.21 ± 0.05 | 10%↑ $q$ = 0.03 | 4.08 ± 0.13 | NS |
| **C20:0** | **Arachidic Acid** | 0.21 ± 0.01 | 0.27 ± 0.01 | 29%↑ $q$ = 0.02 | 0.20 ± 0.02 | NS |
| **C21:0** | **Heneicosylic Acid** | 0.03 ± 0.002 | 0.03 ± 0.003 | NS | 0.02 ± 0.002 | NS |
| **C22:0** | **Behenic Acid** | 0.08 ± 0.003 | 0.09 ± 0.01 | NS | 0.09 ± 0.003 | NS |
| **C23:0** | **Tricosylic Acid** | 0.05 ± 0.003 | 0.04 ± 0.01 | NS | 0.04 ± 0.002 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$).

**Table 3.5 Saturated fatty acid content (% of fat) of milk at day 28 of lactation.**

| Fatty acid | Common name | Control ($n$ = 5) | Canola ($n$ = 5) | Effect (% change) | Sunflower ($n$ = 5) | Effect (% change) |
|---|---|---|---|---|---|---|
| **C4:0** | **Butyric Acid** | 2.34 ± 0.12 | 2.51 ± 0.07 | NS | 2.36 ± 0.07 | NS |
| **C6:0** | **Caproic Acid** | 2.16 ± 0.07 | 2.08 ± 0.01 | NS | 1.99 ± 0.05 | NS |
| **C8:0** | **Caprylic Acid** | 1.99 ± 0.04 | 1.69 ± 0.11 | NS | 1.69 ± 0.03 | 15%↓ $q$ = 0.003 |
| **C10:0** | **Capric Acid** | 3.26 ± 0.04 | 2.63 ± 0.13 | 19%↓ $q$ = 0.01 | 2.72 ± 0.04 | 17%↓ $q$ = 0.0002 |
| **C11:0** | **Undecylic Acid** | 0.12 ± 0.02 | 0.07 ± 0.02 | NS | 0.09 ± 0.02 | NS |
| **C12:0** | **Lauric Acid** | 2.56 ± 0.07 | 1.92 ± 0.09 | 25%↓ $q$ = 0.01 | 2.00 ± 0.03 | 22%↓ $q$ = 0.001 |
| **C13:0** | **Tridecylic Acid** | 0.13 ± 0.02 | 0.09 ± 0.01 | NS | 0.11 ± 0.02 | NS |
| **C14:0** | **Myristic Acid** | 3.54 ± 0.08 | 3.13 ± 0.06 | 12%↓ $q$ = 0.01 | 3.12 ± 0.07 | 12%↓ $q$ = 0.01 |
| **C15:0** | **Pentadecylic Acid** | 0.92 ± 0.06 | 0.77 ± 0.05 | NS | 0.82 ± 0.06 | NS |
| **C16:0** | **Palmitic Acid** | 4.81 ± 0.07 | 4.51 ± 0.05 | 6%↓ $q$ = 0.02 | 4.50 ± 0.06 | 6%↓ $q$= 0.04 |
| **C17:0** | **Margaric Acid** | 0.76 ± 0.03 | 0.77 ± 0.03 | NS | 0.73 ± 0.03 | NS |
| **C18:0** | **Stearic Acid** | 3.39 ± 0.14 | 3.95 ± 0.07 | 17%↑ $q$ = 0.02 | 3.92 ± 0.06 | 16%↑ $q$ = 0.04 |
| **C20:0** | **Arachidic Acid** | 0.25 ± 0.01 | 0.36 ± 0.02 | 44% ↑ $q$ = 0.01 | 0.30 ± 0.01 | NS |
| **C21:0** | **Heneicosylic Acid** | 0.06 ± 0.01 | 0.05 ± 0.01 | NS | 0.06 ± 0.005 | NS |
| **C22:0** | **Behenic Acid** | 0.12 ± 0.01 | 0.13 ± 0.01 | NS | 0.18 ± 0.01 | 50%↑ $q$ = 0.04 |
| **C23:0** | **Tricosylic Acid** | 0.08 ± 0.01 | 0.07 ± 0.01 | NS | 0.08 ± 0.008 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$).

### 3.4.1.3 Monounsaturated fatty acids

Maternal supplementation with canola oil or sunflower oil showed an increase in milk MUFAs. At day 7 of lactation, milk obtained from ewes that received a supplement of canola oil showed a significant 26% increase ($q$ = 0.0002, Figure 3.5). Similarly, a 23% increase was found in the milk of ewes from the sunflower group; however, the increase was not significant ($q$ = 0.06), which may be due to low sample size ($n$ = 3). By day 28 of lactation, both supplemented groups showed an increase of milk MUFAs, by 48% in the canola group ($q$ = 0.0016) and 41% in the sunflower group ($q$ = 0.002).

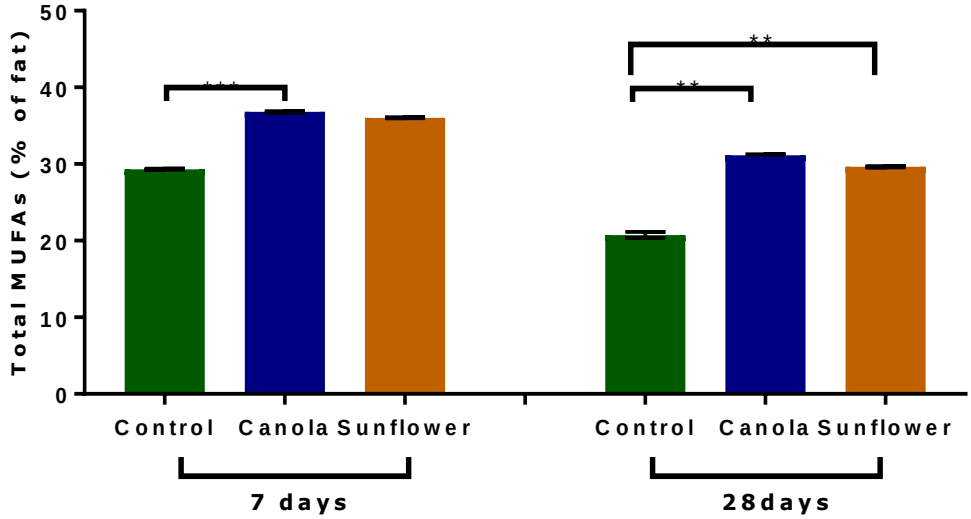



**Figure 3.5 Monounsaturated fatty acid (MUFA) content (% of fat) of milk at days 7 and 28 of lactation.**

Milk samples were taken from ewes fed a standard diet (Control: day 7, $n$ = 5; day 28, $n$ = 5), a standard diet + 3% canola oil (Canola: day 7, $n$ = 5; day 28, $n$ = 5) and a standard diet + 3% sunflower oil (Sunflower: day 7, $n$ = 3; day 28, $n$ = 5). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Significance was denoted by ** $q \leq 0.01$, *** $q \leq 0.001$.

With regard to the analysis of individual MUFAs, data for milk at days 7 and 28 of lactation are shown in Table 3.6 and 3.7, respectively.

The data show that oleic acid (C18:1 n-9) constituted the vast majority of MUFAs with a very close abundant to the total MUFAs. It reveals a significant increase in ewes' milk supplemented with canola oil at both time points: day 7 (24%, $q = 0.007$) and day 28 (53%, $q < 0.0001$) of lactation. In the sunflower-supplemented group's milk, the oleic acid content was higher at day 7 compared with the control; however, it was not statistically significant, again likely due to the low sample size. Yet, by day 28 of lactation, the oleic acid content had significantly increased (45%, $q < 0.0001$). Meanwhile, eicosenoic acid (C20:1 n-9) was significantly increased in ewes' milk in the canola group, but not in the sunflower group, by 50% ($q = 0.03$) at day 7 and 89% ($q = 0.003$) at day 28 of lactation. Erucic acid (C22:1 n-9) content in the supplemented groups showed no significant variation from the control group at either time point, except for a 33% increase ($q = 0.01$) in milk from the canola-supplemented group at day 28 of lactation. Nervonic acid (C24:1 n-9) exhibited no significant differences between the supplemented groups at either time point.

**Table 3.6 Omega-9 monounsaturated fatty acid content (% of fat) of milk at day 7 of lactation.**

| Fatty acid | Common name | Control ($n$ = 5) | Canola ($n$ = 5) | Effect (% change) | Sunflower ($n$ = 3) | Effect (% change) |
|---|---|---|---|---|---|---|
| **C18:1 n-9** | **Oleic Acid** | 29.25 ± 1.7 | 36.72 ± 0.8 | 26%↑ $q$ =0.007 | 35.98 ± 2.7 | NS |
| **C20:1 n-9** | **Eicosenoic Acid** | 0.08 ± 0.004 | 0.12 ± 0.007 | 50%↑ $q$ = 0.03 | 0.09 ± 0.004 | NS |
| **C22:1 n-9** | **Erucic Acid** | 0.03 ± 0.007 | 0.02 ± 0.002 | NS | 0.01 ± 0.0002 | NS |
| **C24:1 n-9** | **Nervonic Acid** | 0.02 ± 0.002 | 0.02 ± 0.002 | NS | 0.01 ± 0.002 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$).

**Table 3.7 Omega-9 monounsaturated fatty acid content (% of fat) of milk at day 28 of lactation.**

| Fatty acid | Common name | Control ($n$ = 5) | Canola ($n$ = 5) | Effect (% change) | Sunflower ($n$ = 5) | Effect (% change) |
|---|---|---|---|---|---|---|
| **C18:1 n-9** | **Oleic Acid** | 20.34 ± 2.4 | 31.03 ± 3.5 | 53%↑ $q$ <0.0001 | 29.58 ± 2.6 | 45%↑ < 0.0001 |
| **C20:1 n-9** | **Eicosenoic Acid** | 0.09 ± 0.006 | 0.17 ± 0.011 | 89%↑ $q$ = 0.003 | 0.11 ± 0.01 | NS |
| **C22:1 n-9** | **Erucic Acid** | 0.03 ± 0.001 | 0.04 ± 0.003 | 33%↑ $q$ = 0.01 | 0.03 ± 0.003 | NS |
| **C24:1 n-9** | **Nervonic Acid** | 0.69 ±0.0004 | 0.03 ± 0.004 | NS | 0.02 ± 0.001 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$).

#### 3.4.1.4 Omega-3 fatty acids

The milk content of ω-3 at days 7 and 28 of lactation is shown in Tables 3.8 and 3.9, respectively. Supplementing ewes' diet with canola oil or sunflower oil did not alter their milk's ω-3 FA abundance at either day 7 or 28.

**Table 3.8 Omega-3 fatty acid content (% of fat) OF milk at day 7 of lactation.**

| Fatty acid | Common name | Control (*n* = 5) | Canola (*n* = 5) | Effect (% change) | Sunflower (*n* = 3) | Effect (% change) |
|---|---|---|---|---|---|---|
| **n-3 PUFA** | **Omega-3 PUFAs** | 1.49 ± 0.077 | 1.09 ± 0.044 | NS | 0.85 ± 0.35 | NS |
| **n-3 LCPUFA** | **Omega-3 LCPUFAs** | 0.52 ± 0.017 | 0.43 ± 0.016 | NS | 0.27 ± 0.11 | NS |
| **C18:3 n-3** | **α-Linolenic Acid (ALA)** | 0.64 ± 0.061 | 0.47 ± 0.033 | NS | 0.69 ± 0.04 | NS |
| **C18:4 n-3** | **Stearidonic Acid** | 0.32 ± 0.035 | 0.20 ± 0.031 | NS | 0.28 ± 0.03 | NS |
| **C20:3 n-3** | **Eicosatrienoic Acid** | 0.01 ± 0.002 | 0.01 ± 0.001 | NS | 0.01 ± 0.0002 | NS |
| **C20:5 n-3** | **Eicosapentaenoic Acid (EPA)** | 0.13 ± 0.007 | 0.11 ± 0.009 | NS | 0.12 ± 0.01 | NS |
| **C22:5 n-3** | **Docosapentaenoic Acid** | 0.27 ± 0.012 | 0.24 ± 0.010 | NS | 0.23 ± 0.01 | NS |
| **C22:6 n-3** | **Docosahexenoic Acid (DHA)** | 0.12 ± 0.009 | 0.07 ± 0.010 | NS | 0.09 ± 0.02 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$). PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid.

**Table 3.9 Omega-3 fatty acid content (% of fat) of milk at day 28 of lactation.**

| Fatty acid | Common name | Control (*n* = 5) | Canola (*n* = 5) | Effect (% change) | Sunflower (*n* = 5) | Effect (% change) |
|---|---|---|---|---|---|---|
| **n-3 PUFA** | **Omega-3 PUFAs** | 1.09 ± 0.044 | 1.17 ± 0.105 | NS | 1.25 ± 0.09 | NS |
| **n-3 LCPUFA** | **Omega-3 LCPUFAs** | 0.43 ± 0.016 | 0.45 ± 0.031 | NS | 0.47 ± 0.03 | NS |
| **C18:3 n-3** | **α-Linolenic Acid (ALA)** | 0.47 ± 0.033 | 0.52 ± 0.071 | NS | 0.46 ± 0.05 | NS |
| **C18:4 n-3** | **Stearidonic Acid** | 0.20 ± 0.031 | 0.21 ± 0.011 | NS | 0.32 ± 0.05 | NS |
| **C20:3 n-3** | **Eicosatrienoic Acid** | 0.01 ± 0.001 | 0.01 ± 0.002 | NS | 0.01 ± 0.001 | NS |
| **C20:5 n-3** | **Eicosapentaenoic Acid (EPA)** | 0.11 ± 0.009 | 0.11 ± 0.011 | NS | 0.11 ± 0.01 | NS |
| **C22:5 n-3** | **Docosapentaenoic Acid** | 0.24 ± 0.010 | 0.25 ± 0.020 | NS | 0.27 ± 0.01 | NS |
| **C22:6 n-3** | **Docosahexenoic Acid (DHA)** | 0.07 ± 0.010 | 0.08 ± 0.004 | NS | 0.08 ± 0.01 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($Q \geq 0.05$). PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid.

#### 3.4.1.5 Omega-6 fatty acids

The milk content of ω-6 at days 7 and 28 of lactation is shown in Tables 3.10 and 3.11, respectively.

Supplementing maternal diet with canola oil had no impact on the total ω-6 PUFAs at either day 7 or day 28 of lactation. However, sunflower oil supplementation did show a significant increase in total ω-6 PUFAs at day 28, but not at day 7, by 12% ($q$ = 0.04). This result can be attributed to changes in the abundance of LA (C18:2 n-6), which constitutes the most abundant ω-6 PUFA, and which showed a significant increase in the sunflower group at day 28 (15%, $q$ = 0.03); unlike canola oil, which did not demonstrate an increase at either time point.

The ω-6 long-chain PUFAs are FAs with 20 or more carbons that are synthesised from linoleic acid (Sprecher, 1999). In contrast to ω-6 PUFAs, ω-6 long-chain PUFAs showed a significant reduction in both intervention groups, by 26% with canola oil ($q$ = 0.01) and 51% with sunflower oil ($q$ = 0.03), at day 7 of lactation. By day 28, only milk supplemented with canola oil showed a decrease in ω-6 long-chain PUFAs (23%, $q$ = 0.003).

The abundance of arachidonic acid (C20:4 n-6) in milk declined significantly in both intervention groups compared with the control by day 7 of lactation, by 22% ($q$ = 0.02) in the canola group and 18% ($q$ = 0.04) in the sunflower group. By day 28, the decline continued in the canola group, but not in the sunflower group (18%, $p$ = 0.03).

Docosadienoic acid (C22:2 n-6) concentrations were significantly decreased in the canola group ewes' milk at day 7 (57%, $q$ = 0.03) and continued to decrease to 75% ($q$ = 0.002) compared with the control by day 28 of lactation. In the sunflower group, C22:2 n-6 concentrations decreased by one-half compared with the control (50%, $q$ = 0.02) at day 28.

The following ω-6 FAs were unaffected by either canola oil or sunflower oil supplementation at both time points: eicosadienoic acid (C20:2 n-6), dihomo-γ-linolenic acid (C20:3 n-6) and adrenic acid (C22:4 n-6).

Finally, γ-linolenic acid (C18:3 n-6) showed a significant reduction only in the milk of the canola-supplemented group at day 28 of lactation, by 43% ($q$ = 0.01), while docosapentaenoic acid (C22:5 n-6) concentrations were significantly lower only in the sunflower-supplemented group's milk (90%, $q$ = 0.01) at day 7 when compared to the control.

**Table 3.10 Omega-6 fatty acid content (% of fat) of milk at day 7 of lactation.**

| Fatty acid | Common name | Control (*n* = 5) | Canola (*n* = 5) | Effect (% change) | Sunflower (*n* = 3) | Effect (% change) |
|---|---|---|---|---|---|---|
| **n-6 PUFA** | **Omega-6 PUFAs** | 2.08 ± 0.053 | 1.88 ± 0.0916 | NS | 1.25 ± 0.51 | NS |
| **n-6 LCPUFA** | **Omega-6 LCPUFAs** | 0.35 ± 0.007 | 0.26 ± 3.027 | 26%↓ $q$ = 0.01 | 0.17 ± 0.07 | 51%↓ $q$ = 0.03 |
| **C18:2 n-6** | **Linoleic Acid (LA)** | 1.69 ± 0.0544 | 1.59 ± 6.071 | NS | 1.80 ± 0.10 | NS |
| **C18:3 n-6** | **γ-linolenic Acid** | 0.05 ± 0.0047 | 0.03 ± 0.002 | NS | 0.04 ± 0.005 | NS |
| **C20:2 n-6** | **Eicosadienoic Acid** | 0.07 ± 0.0035 | 0.06 ± 0.006 | NS | 0.06 ± 0.01 | NS |
| **C20:3 n-6** | **Dihomo-γ-linolenic Acid** | 0.02 ± 0.0011 | 0.02 ± 0.003 | NS | 0.02 ± 0.002 | NS |
| **C20:4 n-6** | **Arachidonic Acid (AA)** | 0.23 ± 0.0085 | 0.18 ± 2.019 | 22%↓ $q$ = 0.02 | 0.19 ± 0.001 | 18%↓ $q$ = 0.04 |
| **C22:2 n-6** | **Docosadienoic Acid** | 0.0007 ± 0.0001 | 0.0003 ± 0.0001 | 57%↓ $q$ = 0.03 | 0.0004 ± 0.0001 | NS |
| **C22:4 n-6** | **Adrenic Acid** | 0.01 ± 0.0004 | 0.005 ± 0.0005 | NS | 0.004 ± 0.001 | NS |
| **C22:5 n-6** | **Docosapentaenoic Acid** | 0.01 ± 0.0002 | 0.001 ± 0.0005 | NS | 0.001 ± 0.001 | 90%↓ $q$ = 0.01 |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$). PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid. NA= data not available.

**Table 3.11 Omega-6 fatty acid content (% of fat) of milk at day 28 of lactation.**

| Fatty acid | Common name | Control (*n* = 5) | Canola (*n* = 5) | Effect (% change) | Sunflower (*n* = 5) | Effect (% change) |
|---|---|---|---|---|---|---|
| **n-6 PUFA** | **Omega-6 PUFAs** | 2.10 ± 0.063 | 1.95 ± 0.094 | NS | 2.36 ± 0.09 | 12%↑ $q$ = 0.04 |
| **n-6 LCPUFA** | **Omega-6 LCPUFAs** | 0.39 ± 0.013 | 0.30 ± 0.018 | 23%↓ $q$ = 0.003 | 0.41 ± 0.03 | NS |
| **C18:2 n-6** | **Linoleic Acid (LA)** | 1.63 ± 0.064 | 1.62 ± 6.084 | NS | 1.88 ± 0.05 | 15%↑ $q$ = 0.03 |
| **C18:3 n-6** | **γ-linolenic Acid** | 0.07 ± 0.003 | 0.04 ± 0.006 | 43%↓ $q$ = 0.01 | 0.07 ± 0.01 | NS |
| **C20:2 n-6** | **Eicosadienoic Acid** | 0.11 ± 0.009 | 0.08 ± 0.008 | NS | 0.11 ± 0.01 | NS |
| **C20:3 n-6** | **Dihomo-γ-linolenic Acid** | 0.03 ± 0.002 | 0.03 ± 0.004 | NS | 0.04 ± 0.003 | NS |
| **C20:4 n-6** | **Arachidonic Acid (AA)** | 0.22 ± 0.009 | 0.18 ± 2.008 | 18%↓ $q$ = 0.03 | 0.24 ± 0.02 | NS |
| **C22:2 n-6** | **Docosadienoic Acid** | 0.002 ± 0.0002 | 0.0005 ± 0.0001 | 75%↓ $q$ = 0.002 | 0.001 ± 0.0002 | 50%↓ $q$ = 0.02 |
| **C22:4 n-6** | **Adrenic Acid** | 0.02 ± 0.001 | 0.01 ± 0.01 | NS | 0.02 ± 0.003 | NS |
| **C22:5 n-6** | **Docosapentaenoic Acid** | 0.00 ± 0.001 | 0.00 ± 0.00 | NS | 0.007 ± 0.001 | NS |

Milk samples were taken from ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) and a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$). PUFA = polyunsaturated fatty acid. LCPUFA = long-chain polyunsaturated fatty acid.

### 3.4.1.6 *Trans* fatty acids

Supplementing maternal diet with canola oil or sunflower oil increased the TFA content of milk at day 7 of lactation by 59% ($q$ = 0.0001) and 37% ($q$ = 0.002), respectively, as shown in Figure 3.6. It is likely that the reason for the smaller increase in the TFA content in the sunflower group than in the canola group was the small size of the sunflower group ($n$ = 3).

By day 28 of lactation, TFA content had continued to increase, reaching 114% in the canola group and 146% in the sunflower group (both $q$ = 0.0001) compared to the control.

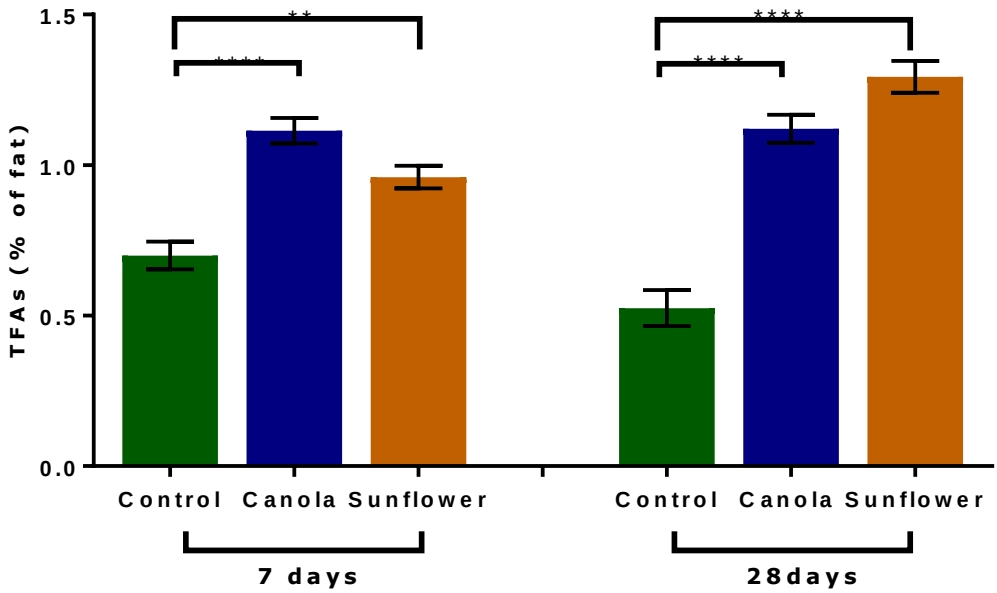



**Figure 3.6 *Trans* fatty acid content (% of fat) of milk at days 7 and 28 of lactation.**

Milk samples were taken from ewes fed a standard diet (Control: day 7, $n$ = 5; day 28, $n$ = 5), a standard diet + 3% canola oil (Canola: day 7, $n$ = 5; day 28, $n$ = 5) and a standard diet + 3% sunflower oil (Sunflower: day 7, $n$ = 3; day 28, $n$ = 5). Values are mean ± SEM. Within a day's sampling, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. Significance was denoted by ** $q \leq 0.01$, **** $q \leq 0.0001$.

### 3.4.2 The effects of supplementing ewes' diet with fatty acids on their body weight

Supplementing ewes' diet with canola oil or sunflower oil had no effect on their body weight at either day 7 or day 28 of lactation, as shown in Figure 3.7.

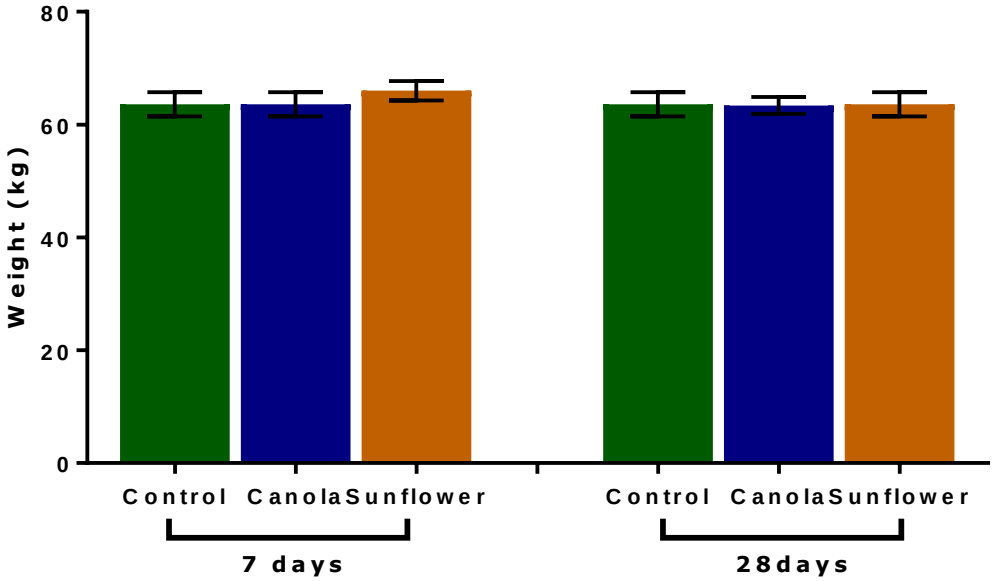



**Figure 3.7 Ewe body weight (kg) by dietary group at days 7 and 28 of lactation.**

Body weight was measured for ewes fed a standard diet (Control: day 7, *n* = 5; day 28, *n* = 5), a standard diet + 3% canola oil (Canola: day 7, *n* = 5; day 28, *n* = 5) and a standard diet + 3% sunflower oil (Sunflower: day 7, *n* = 3; day 28, *n* = 5). Values are mean ± SEM. Within a day's weighting, the mean of each intervention group was compared with that of the control group by one-way ANOVA and FDR post hoc test. There were no significant differences ($q \geq 0.05$).

## 3.5 Discussion

A summary of the effects of the supplements on milk FA profiles is provided in Table 3.12.

**Table 3.12 Summary of changes induced in milk fatty acid profiles at days 7 and 28 with maternal dietary supplements of either 3% canola or 3% sunflower oil.**

| | | | Day 7 | | Day 28 | |
|---|---|---|---|---|---|---|
| | | | Canola | Sunflower | Canola | Sunflower |
| **Total fat** | | | ↔ | ↔ | ↔ | ↔ |
| **Saturated fatty acids** | short | C4:0 | ↔ | ↔ | ↔ | ↔ |
| | medium | C6:0 | ↓ | ↔ | ↔ | ↔ |
| | | C8:0 | ↓ | ↔ | ↔ | ↓ |
| | | C10:0 | ↓ | ↔ | ↓ | ↓ |
| | | C11:0 | ↓ | ↔ | ↔ | ↔ |
| | | C12:0 | ↓ | ↔ | ↓ | ↓ |
| | long | C13:0 | ↔ | ↔ | ↔ | ↔ |
| | | C14:0 | ↓ | ↔ | ↓ | ↓ |
| | | C15:0 | ↔ | ↔ | ↔ | ↔ |
| | | C16:0 | ↓ | ↔ | ↓ | ↓ |
| | | C17:0 | ↔ | ↔ | ↔ | ↔ |
| | | C18:0 | ↑ | ↔ | ↑ | ↑ |
| | | C20:0 | ↑ | ↔ | ↑ | ↔ |
| | | C21:0 | ↔ | ↔ | ↔ | ↔ |
| | V.long | C22:0 | ↔ | ↔ | ↔ | ↑ |
| | | C23:0 | ↔ | ↔ | ↔ | ↔ |
| **Monounsaturated fatty acids** | | C18:1 n-9 | ↑ | ↔ | ↑ | ↑ |
| | | C20:1 n-9 | ↑ | ↔ | ↑ | ↔ |
| | | C22:1 n-9 | ↔ | ↔ | ↑ | ↔ |
| | | C24:1 n-9 | ↔ | ↔ | ↔ | ↔ |
| **omega-3 fatty acids** | | | ↔ | ↔ | ↔ | ↔ |
| **omega-6 fatty acids** | | n-6 PUFA | ↔ | ↔ | ↔ | ↑ |
| | | n-6 LCPUFA | ↓ | ↓ | ↓ | ↔ |
| | | C18:2 n-6 | ↔ | ↔ | ↔ | ↑ |
| | | C18:3 n-6 | ↔ | ↔ | ↓ | ↔ |
| | | C20:2 n-6 | ↔ | ↔ | ↔ | ↔ |
| | | C20:3 n-6 | ↔ | ↔ | ↔ | ↔ |
| | | C20:4 n-6 | ↓ | ↓ | ↓ | ↔ |
| | | C22:2 n-6 | ↓ | ↔ | ↓ | ↓ |
| | | C22:4 n-6 | ↔ | ↔ | ↔ | ↔ |
| | | C22:5 n-6 | ↔ | ↓ | ↔ | ↔ |
| ***Trans* fat** | | | ↑ | ↑ | ↑ | ↑ |

Symbols indicate the difference in content compared with the control: ↔ = no change, ↑ = increase and ↓ = decrease. Short = short-chain saturated fatty acids. Medium = medium-chain saturated fatty acids. Long = long-chain saturated fatty acids. V.long = very long-chain saturated fatty acids. PUFA = polyunsaturated fatty acids. LCPUFA = long-chain polyunsaturated fatty acids.

### 3.5.1 Fatty acid supplementation had no effect on total milk fat content

There was no effect of supplementations observed on the total levels of milk fat at either day 7 or 28 of lactation. Although many studies have been conducted on mammals to investigate the effect of diet on the fat content of milk, the results were conflicting. Similar to this investigation's findings, DePeters et al. (2001) found that the fat content of cows' milk was not affected by supplementing their diet with 1.6% canola oil. Other studies have demonstrated that fat is the most variable component in milk content, either decreasing in cows' milk after 3.5% canola oil supplementation (Jenkins et al., 1998) or steadily increasing in goats with 2%, 4% and 6% canola oil supplementation (Mir et al., 1999). In a similar manner, supplementing ewes' diets with either 2% (Toral et al., 2010a) or 2.5% (Toral et al., 2010b) sunflower oil had no effect on the total fat content in their milk, whilst supplementing sheep with 6% sunflower oil caused an increase in milk fat (Hervás et al., 2008). In contrast, the fat content of cow's milk was reduced when diets were supplemented with sunflower oil and fish oil over time (Shingfield et al., 2006). It thus appears that the effects of supplements are dependent on the given doses.

The contradictory results of studies conducted on ruminants on the effect of diet on milk fat content highlights other potentially contributory factors, such as seasonal variations (Lock & Garnsworthy, 2003; Saroj et al., 2016). Lock and Garnsworthy (2003) reported variations in milk fat concentrations in cows between season, with the highest milk fat levels occurring in the summer (May) and the lowest in the winter (December/February). This pattern could be attributed to the seasonal availability of green foliage. Conversely, other studies reported the highest milk fat levels during the winter and the lowest during the summer (Heck et al., 2009; Ozrenk & Inci, 2008), which may be attributable to the level of prolactin release, whose concentrations in plasma are higher in the summer due to the higher light-to-dark ratio (Sevi et al., 2004) resulting in produce greater volumes of milk with lower fat content (Marcek & Swanson, 1984). Moreover, a low fibre and high grain diet, which is more typical in

the winter, also increases milk fat (Waldner et al., 2005). On the other hand, seasonal effects on milk fat content were not significant (Saroj et al., 2016) or showed irregular trends and were different across sheep flocks (Carta et al., 1995) in other studies.

### 3.5.2 Maternal supplementation with canola and sunflower oil had effects on saturated fatty acid content of milk

FA content of milk from ewes fed the control diet was similar to that reported by Jandal (1996), although, the C10:0, C14:0, C16:0 and C18:0 contents were lower. These differences in FA content could be attributed to a range of factors including diet, breed, individual traits and period of lactation (Jenness, 1980; Markiewicz-Kęszycka et al., 2013; Okine et al., 2003). It is noteworthy that even though substantial research has been conducted on ruminants' milk, studies in sheep remain scarce (Toral et al., 2010).

Overall, a decrease in medium-chain SFAs and an increase in long-chain SFAs in milk were observed with either canola oil or sunflower oil supplementation. The decline in medium-chain SFA concentration was consistent with the results of studies performed on ruminants that showed that canola oil supplementation of maternal diet significantly decreased medium-chain SFA concentration in milk (Mir et al., 2000; Okine et al., 2003; Vafa et al., 2012; Welter et al, 2016). The same effect was achieved with sunflower oil supplementation (Hervás et al., 2008; Rego et al., 2009). It is well established that an increased supply of LCFAs to the mammary gland modifies the synthesis of short- and medium-chain SFAs (Chilliard et al., 2007; Kadegowda et al., 2009; Pulina et al., 2006). Increasing dietary LCFAs (e.g. by supplementing the diet with canola oil or sunflower oil) regulates in part the different lipogenic pathways (including *de novo* synthesis in the mammary gland), which in turn results in decreased *de novo* FA synthesis (Barber et al., 1997; Chilliard et al., 1991; Chilliard et al., 2000; Hansen et al., 1986, 1987). Because high consumption of SFAs is a potential risk factor for plasmatic cholesterol and obesity (Ohlsson, 2010; Zambiazi et al., 2007) as well as cardiovascular disease and metabolic syndrome

(Shingfield et al., 2008), the effect of canola oil and sunflower oil supplementation on the nutritional value of milk fat would be positive on a range of health outcomes. On the other hand, the putative effect of a specific SFA on human health should be considered individually (Shingfield et al., 2008) as there are some exceptions.

Lauric acid (C12:0) and myristic acid (C14:0) were reported to have probable negative effects on lipoprotein concentrations and serum HDL cholesterol in adults (Sundram et al., 1994; Zock et al., 1994). In infants, it has been recommended that the total amount of myristic acid and lauric acid should be less than 20% of the total fat in infant formula (Koletzko et al., 2005). These results indicate that the decreases in lauric acid and myristic acid observed in this study are potentially advantageous in terms of infant and adult health.

#### 3.5.2.1 Canola and sunflower oil supplementation during lactation decreases palmitic acid (C16:0) in milk

With regard to the effect of supplementing ewes' diet with canola or sunflower oil on LCFA concentrations in milk, there was a small (6%) yet significant decrease in palmitic acid in both the canola and sunflower groups. This result is similar to what Ashes et al. (1992) observed (6.9% decrease in palmitic acid) in their manipulation of the FA composition of milk by feeding canola seeds to goats. Palmitic acid is the main SFA in the milk fat of the majority of mammals (Markiewicz-Kęszycka et al., 2013) and about 50% of this SFA is synthesised by mammary gland *de novo* (Chilliard et al., 2000), which is responsible for the synthesis of FAs up to 16 carbons in length (Bauman et al., 2011). Plant oil supplements could shift FA synthesis towards 18:0, at the expense of 16:0, due to the inhibition of LCFAs for *de novo* FA synthesis (Chilliard et al., 2000). Reducing palmitic acid in milk with dietary supplements may be particularly beneficial in light of the suggestion by the WHO in 2003 that there was convincing evidence that palmitic acid increased the risk of CVD (WHO, 2003). In adults, diets rich in SFAs, such as lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0), have been reported to be hypercholesteraemic (Bonanome &

Grundy, 1988). However, palmitic acid has been shown to play a role in inducing lower LDL cholesterol by 11% when consumed instead of lauric and myristic acid (Sundram et al., 1994); thus, palmitic acid appears to be more beneficial to consume than some other SFAs. However, the consequences of palmitic acid consumption for infant health are different than they are for adult health. Palmitic acid is present in human breast milk, at about 23% (German & Dillard, 2010), and many studies have shown that it is likely to have important nutritional and biological effects, including improving intestinal absorption of calcium (Carnielli et al., 1995, 1996), promoting lipoprotein metabolism (Lai et al., 2008), and aiding in metabolic regulation (Wareski et al., 2009). It also comprises about one-half of the FAs in an infant's adipose tissue (Baker, 1969; Boersma, 1979; Farquharson et al., 1993; Sweeney et al., 1963) and contributes about 10% of the total daily energy (Innis, 2013). From the above, it can be said that the health consequences for adults of consuming palmitic acid cannot be generalised to infants, especially considering that FA biosynthesis in milk is a complex process involving many metabolic pathways and happens at different stages of development of the mammary gland (Smoczyński et al., 2012).

#### 3.5.2.2 Canola and sunflower oil supplementation during lactation increases stearic acid (C18:0) in milk

Stearic acid (C18:0) has shown comparable increases in goats' (Okien et al., 2003) and cows' (Welter et al., 20.16) milk when supplemented with canola oil. It has also been demonstrated that oilseeds rich in USFAs (such as canola and sunflower) increase LCFAs at the expense of medium-chain FAs, such as C14:0 and C16:0, in cows' milk (Khorasani et al., 1991). When entering the rumen, oleic (C18:1 n-9), linoleic (C18:2n-6) and linolenic (C18:3n-6) UFAs become hydrogenated into stearic acid, the final product of the complete biohydrogenation of linoleic acid (C18:2) (Kemp & Lander, 1983). It is likely that the increase in stearic acid observed in the present study was due to high concentrations of dietary oleic acid (C18:1 n-9), particularly in canola oil (c. 62%), which hydrogenated into stearic acid. Unlike other saturated FAs, ,which all raise low-density lipoprotein (LDL) cholesterol levels (Denke, 2006), C18:0

has been reported to have lowering effects on LDL (Aro et al., 1997; Bonanome & Grundy, 1988) and total cholesterol (Grundy, 1994; Zock & Katan, 1992).

#### 3.5.2.3 Canola oil supplementation during lactation increases arachidic acid (C20:0) in milk

Arachidic acid (C20:0), also called eicosanoic acid, is a long-chain SFA that was also increased with canola oil supplementation in this study. This is similar to Welter et al.'s (2016) findings, which showed a 67% increase in cow's milk arachidic acid after canola oil supplementation. In contrast, no effect of sunflower supplements was observed, which is consistent with other studies (Hervas et al., 2008; Toral et al., 2010). The supplementation of canola oil (C. 93% USFs) in the diet of lactating ewes produced TFAs as a result of incomplete ruminal biohydrogenation of dietary USFs, which are absorbed in the gut and then directed to tissues such as the mammary gland. The expression of lipogenic enzymes that control FA synthesis *de novo* may subsequently decline (Palmquist et al., 2006). As FAs of up to 16 carbons in length are formed *de novo* (Bauman et al., 2011), the decrease observed in FA concentrations up to C16:0 and the linear increase in FAs above C18:0 with canola oil supplements suggest a reduction of lipogenic enzyme activity in *de novo* synthesis (Welter et al., 2016). Moreover, arachidic acid can be formed by the hydrogenation of arachidonic acid (20:4 n-6) (Howton & Mead, 1960), the reduction of which (by 16%) found after canola supplementation suggests it was dehydrogenated into arachidic acid.

### 3.5.3 Monounsaturated fatty acids are increased with canola and, to a lesser extent, sunflower oil supplements

MUFA concentrations increased significantly in both supplemented groups compared to the control. As detailed in Table 3.2, canola oil consists of approximately 26% MUFAs, while sunflower oil has little higher levels of about 37%.In contrast, other studies showed tht canola oil has much higher level of MUFAs copmpared with sunflower oil (Dubois et al., 2007; Zambiazi et al., 2007). However, similar increase seen in milk in both groups at day 28 of lactation (48% in the canola group and 41% in the sunflower

group) suggests that dietary MUFA intake is not the main cause of its increase in milk. It is more likely that increased FA intake causes a reduction in the rate of ruminal biohydrogenation, which in turn results in an increase of MUFAs (Palmquist et al., 2006). The increase observed in MUFAs is similar to what has been seen in other studies. Milk MUFA levels increased by 28% with 3% canola oil supplementation in cows (Welter et al., 2016) and 27% with 4% canola oil supplementation in goats (Okine et al., 2003). Herva et al. (2008) and Toral et al. (2009) found significant changes in almost all MUFAs in milk when the diet was supplemented with sunflower oil. Another study demonstrated an increase in MUFAs with sunflower or rapeseed oil supplements (Rego et al., 2009).

MUFAs account for about one-quarter of the FAs in milk (Mansson, 2008), the most abundant of which is oleic acid (C18:1 n-9) (Mansbridge & Blake, 1997; Mansson, 2008), which accounts for > 95% of milk MUFAs in this study. Oleic acid is formed from stearic acid in the mammary gland by delta-9 desaturation (Chilliard et al., 2000). The increase in oleic acid observed in this study with canola and sunflower supplements can be traced to the decline in the biohydrogenation rate of oleic acid caused by the supplementation. It has been demonstrated that supplementing cows' diet with UFAs reduces the ruminal biohydrogenation of oleic acid (C18:1) (Harvatine & Allen, 2006).

Recently, some studies have demonstrated the beneficial effects of oleic acid on health, such as modulating blood pressure (Terés et al., 2008), promoting weight loss by increasing the expression of genes involved in fat burning (Lim et al., 2013), mitigating the symptoms of type II diabetes (Vassiliou et al., 2009), protecting cell membranes from free radicals and oxidative stress (Haug et al., 2007) and reduce the risk of autoimmune and inflammatory diseases (Sales-Campos et al., 2013). Moreover, oleic acid is helpful in lowering LDL cholesterol (Mattson & Brundy, 1985) and triacyclglycerols, which makes it more effective in preventing heart diseases than other FAs (Rui et al., 2007).

#### 3.5.3.1 Canola oil supplementation increased erucic acid (C22:1 n-9)

Canola oil is a low erucic acid rapeseed (LEAR) oil. Canola oil has a much lower erucic acid content (c. 0.3-0.6%) (FSANZ, 2003) than rapeseed oil (c. 30-50%) (GRAS, 2012) in terms of total FAs. In this investigation, canola, but not sunflower, supplementation caused a 33% increase in erucic acid (C22:1 n-9) in milk at day 28 of lactation. Similarly, Welter et al. (2016) observed an 11% increase in erucic acid in cows fed 3% canola oil. As diet is the usual source of LCFAs, not *de novo* synthesis (Bauman & Griinari, 2003; Harvatine et al., 2009), it is likely that canola oil supplementation (Gunstone, 2004) accounts for the increase in erucic acid observed in the milk in this study.

Many studies on different animal species have confirmed the association between increased erucic acid in diet and myocardial lipidosis (GRAS, 2012). However, there is no epidemiological evidence to suggest an increase in cardiovascular disease among populations that consume oils rich in erucic acid (Aloe & Heggtveit, 1983; Svaar, 1982). According to the European Commission Directive (AECD, 2006) and Food Standards Australia New Zealand (FSANZ, 2003), erucic acid content in infant formula should not exceed 1% of the total fat content. Although erucic acid concentrations were increased in milk obtained from ewes fed canola oil supplements, the percentages were still well within this limit.

### 3.5.4 Omega-3 fatty acids are unaffected by supplementation

No effect was observed on ω-3 FA levels in milk after supplementation at either day 7 or day 28 of lactation. This is identical to Welter et al.'s (2016) findings after supplementing cows' diet with 3% canola oil.

Omega-3 is an essential FA; therefore, it cannot be produced by ruminants and must be obtained from their diet. Nevertheless, ruminants' feed is generally very low in, or free of, ω -3 (Bernal-Santos et al., 2009). Fortunately, ω-3 levels in milk can be affected by changing the diet (Bernal-Santos et al., 2009). Accordingly, it is possible

to enhance ω-3 FAs in milk by increasing the dosage of supplements. For example, increasing canola oil supplements in cow's diet from 3% to 6% resulted in doubled ω-3 FA content (Welter et al., 2016). Moreover,, the inclusion of fish oil (Shingfield et al., 2006) or marine algae/oils (Franklin et al., 1999; Halliard et al., 2000; Herva et al., 2008; Vafa et al., 2012) in existing supplements is likely to be more effective, where it has been proven to increase individual ω-3 FAs.

Long-chain ω-3 FAs, particularly eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), are beneficial for maintaining human health (Bernal-Santos et al., 2009). In adults, many studies have shown EPA /DHA's role in preventing CVD, inflammatory diseases and neurological disorders (Gebauer et al., 2006; Wang et al., 2006; Yashodhara et al., 2009). Dietary supplementation with ω-3 may also benefit people with diabetes (Mori et al., 1999), metabolic syndrome (Esposito et al., 2004) and hypertension (Ueshima et al., 2007). Moreover, ω-3 has the potential to encourage weight loss when consumed as a component of a weight-loss diet, improving oxidative stress markers in obese patients (Parra et al., 2007) and preventing obesity by activating UCP1 (Kim et al., 2015). In infants, dietary DHA increases visual, mental and motor skill development (Innis, 2008, 2009).

### 3.5.5 Maternal supplementation with canola and sunflower oil had opposite effects on omega-6 fatty acids in milk

The effect of canola oil on ω-6 FAs in milk is opposite of that induced by sunflower oil, unlike their otherwise similar effect on the content of other FAs. The ω-6 FAs consist of a group of polyunsaturated fats, of which linoleic acid (LA, C18:2 n-6) and arachidonic acid (AA; C20:4 n-6) are the most important. With canola oil supplementation, ω-6 LCPUFA decreased in milk even though the level of linoleic acid, the most abundant ω-6 FA, was not changed. Likewise, linoleic acid in cows' milk was not affected when their diet was supplemented with 2% or 4% canola oil (Okine et al., 2003). Overall, the decrease observed in arachidonic acid with canola oil supplementation was particularly beneficial, as excess levels have been associated

with increased inflammation and reduced anti-inflammatory effects of ω-3 FAs (Yashodhara et al., 2009). Since ω-6 metabolism can interfere with ω-3 metabolism (Ibeagha-Awemu et al., 2014), the effect of canola oil supplementation in lowering the amount of ω-6 in ewes' milk is particularly promising due to its potential to improve ω-3 metabolism.

In contrast, sunflower oil supplementation increased concentrations of ω-6 FAs in milk. It is likely that the high content of ω-6 via sunflower oil supplementation (FAO, 1999) is responsible for the increase of ω-6 observed in ewes' milk. Although most of the ω-6 FAs were unaffected by sunflower oil supplementation, the increase in linoleic acid levels caused a concomitant increase in total ω-6 FA content. Linoleic acid is an essential FA that cannot be synthesised in the body and must be obtained from food (Burr et al., 1932); and plays a role in maintaining heart health, reducing total and LDL cholesterol, and improving insulin sensitivity and blood pressure (Farvid et al., 2014). On the other hand, a high level of linoleic acid in milk could increase offspring adiposity, as seen in rats (Massiera et al., 2003); and fat mass, adipocyte number and size, as seen in vitro (Azain et al., 2004; Gaillard et al., 1989; Muhlhausler et al., 2010). In infants, due to the immaturity of delta-6-desaturase, the required metabolism of linoleic acid cannot be achieved; therefore, breast milk contains γ-linolenic acid (C18:3 n-6), dihomo-γ-linolenic acid (C20:3 n-6) and arachidonic acid (C20:4 n-6). In contrast, regular infant formula only contains linoleic and α-linolenic acid (C18:3 n-3), which may represent a deficiency in formula fed to infants (Nagano et al., 2016; Novak et al., 2012).

### 3.5.6 *Trans* fatty acids in milk are multiplied with sunflower oil and, to a lesser extent, canola oil supplementation

A substantial increase in TFA levels was observed in both supplemented groups at both time points, especially in the sunflower-supplemented ewes' milk. This increase can be explained in part by the concomitant increase in conjugated linoleic acid (CLA), as investigated by a colleague Dr. Rachel Woods (75% in the canola group and 85% in

the sunflower group) (data not published), which has been classed as the main TFA in ruminants' milk (Markiewicz-Kęszycka et al., 2013). Moreover, the supplements used in this study were high in UFAs (82% and 83% in canola oil and sunflower oil, respectively) and the majority pass through the rumen without or with only partial biohydrogenation of FAs, resulting in the formation of intermediate FAs such as TFAs (Welter et al., 2016).

It is worth mentioning that although there was an increase in milk TFA, it was still much lower than those reported in other sheep studies (Aro et al., 1998; Park & Haenlein, 2013; Talpur et al., 2008). These differences could be attributed to differences between breeds of sheep, which have been shown to influence milk FA profiles (Markiewicz-Kęszycka et al., 2013; Mierlita et al., 2011). For example, TFA content was in the range of 2.59% to 3.59% of total FAs in the milk of Kooka sheep, which are endemic to Pakistan (Talpur et al., 2008); these levels are above the highest TFA levels observed in my investigation, where the TFA content was 1.3% of total FAs in the milk of the sunflower group at day 28 of lactation.

Recently, it has been shown that the natural TFAs in ruminants' milk are beneficial for cardiovascular health (Dilzer & Park, 2012). However, these findings cannot be generalised to early life as TFAs may negatively affect chain elongation, lipoprotein metabolism and growth of essential FAs (Carlson et al., 1997; Koletzko, 1992). Therefore, the European Food Safety Authority (EFSA, 2006) determined the upper limit of TFAs in infant formula as 3% of total fat content, which is commensurate with the TFA levels measured in this study.

## 3.6 Conclusion

FA profiles of milk can be modified over time through changes in an animal's diet, with canola oil and/or sunflower oil supplements demonstrating beneficial results. In this chapter, I hypothesised that supplementing maternal diet with canola or sunflower oils would decrease SFAs, increase MUFAs, and increase TFAs in ewes' milk.

Both dietary supplements resulted in marked changes in milk FA profiles throughout the first month of lactation, which were characterised by a decrease in SFAs up to C16:0, albeit at the expense of an increase in C18:0. Canola oil increased MUFAs to a greater extent than sunflower oil. Both supplements had no effect on ω-3 FAs. With regard to ω-6, canola oil supplementation reduced the majority of these acids with the exception of LA, which was increased by sunflower oil supplementation and thus resulted in an increase in total ω-6 FAs. However, despite the changes in milk FA profiles induced by supplementation, which improved the composition of milk fat, large increases in TFAs were also observed. A summary of the effects of the supplements on milk FA profiles is provided in Figures 3.8-11.

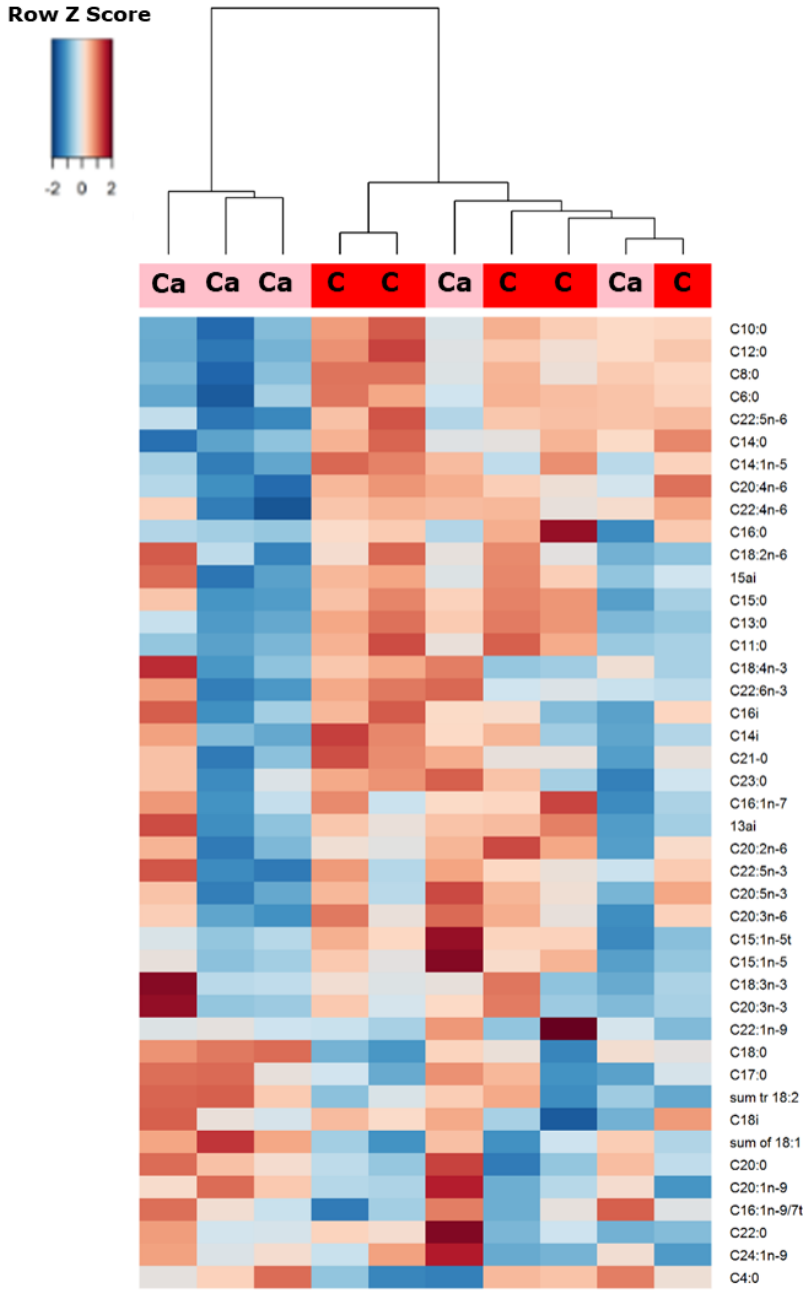



**Figure 3.8 Heat map for milk fatty acid profile at day 7 of lactation (control vs. canola).**

Samples from ewes fed a standard diet (control) or a standard diet + 3% canola oil (canola). Fatty acid content was transformed to a z-score. Fatty acid relative quantities are visualised by a colour scale ranges from bright red (increase) through to dark blue (decrease), see legend. Each rectangle represents an individual sample and is color-coded to indicate the specific dietary group which it belongs (red is C; control, pink is Ca; canola).

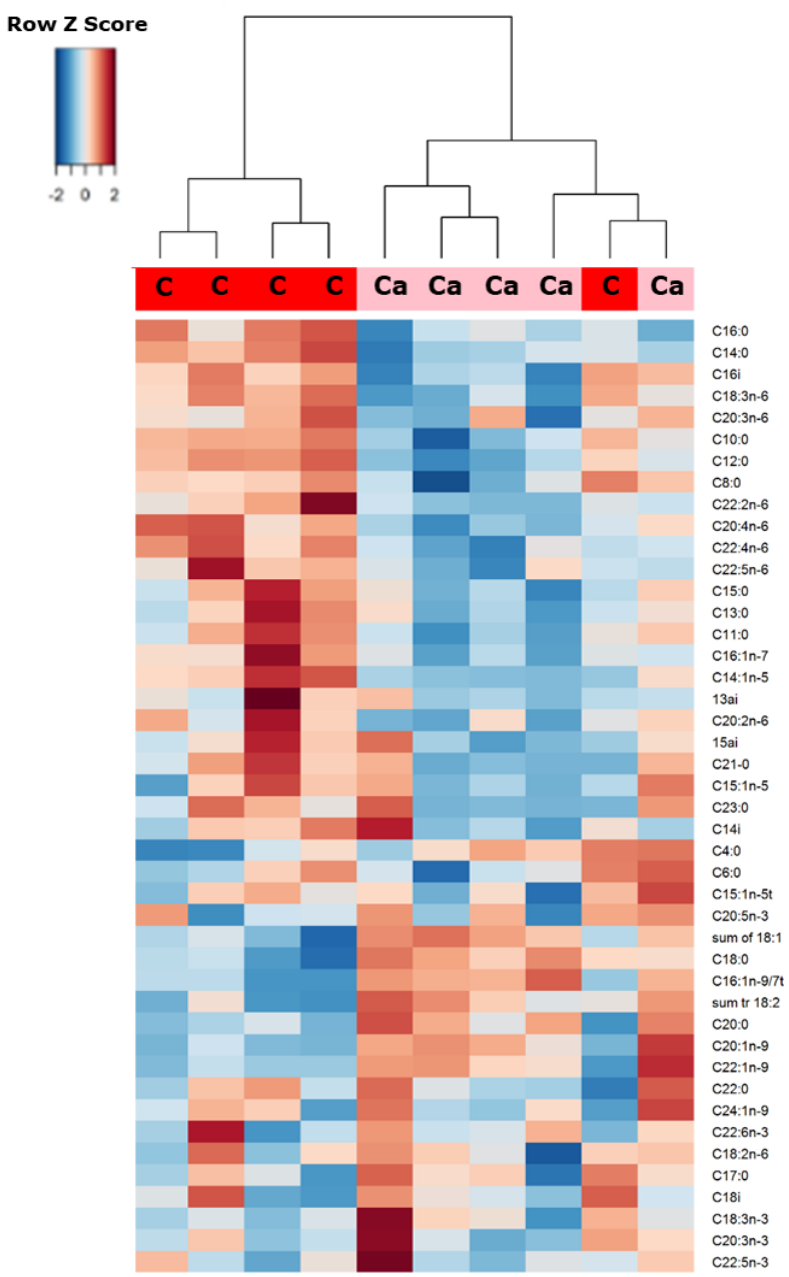



**Figure 3.9 Heat map for milk fatty acid profile at day 28 of lactation (control vs. canola).**

Samples from ewes fed a standard diet (control) or a standard diet + 3% canola oil (canola). Fatty acid content was transformed to a z-score. Fatty acid relative quantities are visualised by a colour scale ranges from bright red (increase) through to dark blue (decrease), see legend. Each rectangle represents an individual sample and is color-coded to indicate the specific dietary group which it belongs (red is C; control, pink is Ca; canola).

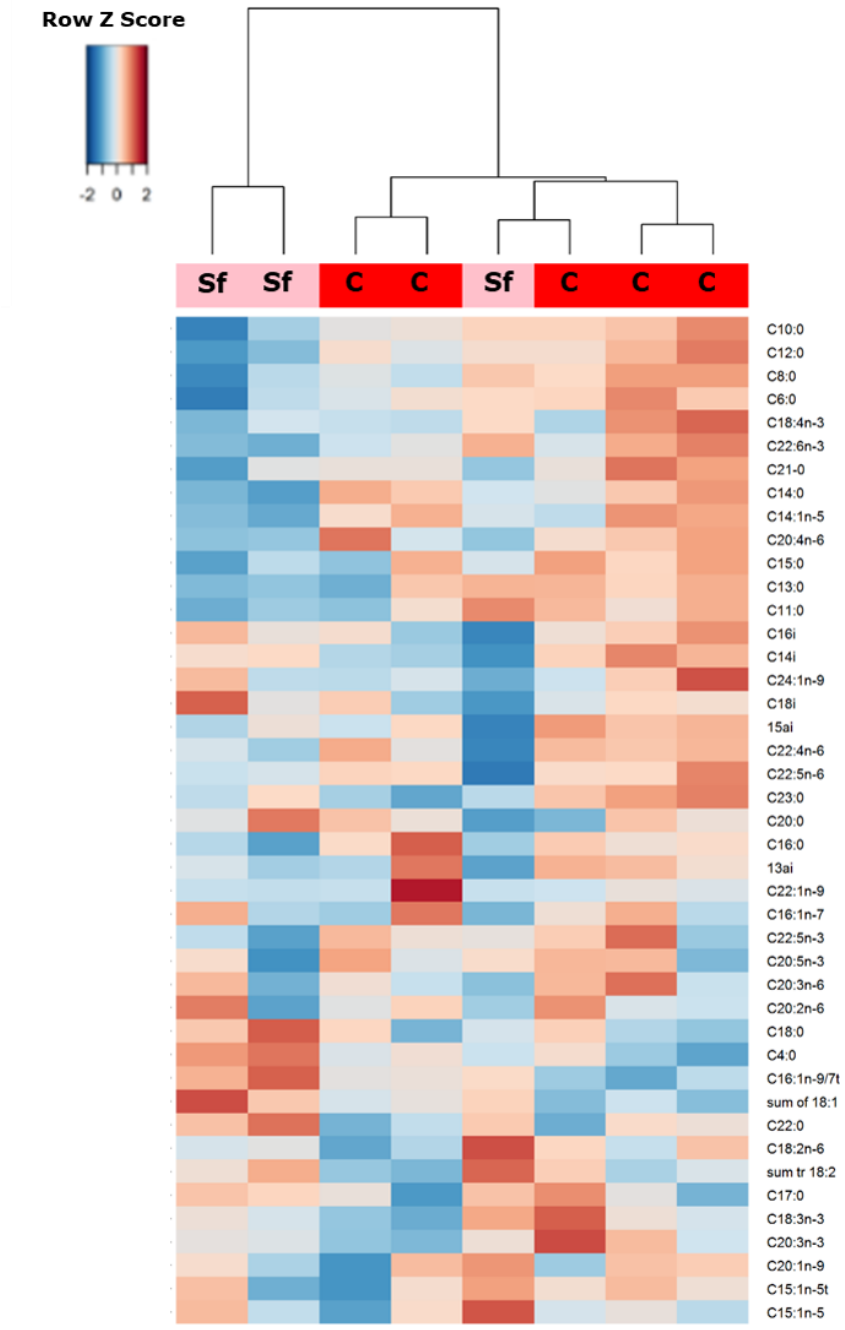



**Figure 3.10 Heat map for milk fatty acid profile at day 7 of lactation (control vs. sunflower).**

Samples from ewes fed a standard diet (control) or a standard diet + 3% sunflower oil (sunflower). Fatty acid content was transformed to a z-score. Fatty acid relative quantities are visualised by a colour scale ranges from bright red (increase) through to dark blue (decrease), see legend. Each rectangle represents an individual sample and is color-coded to indicate the specific dietary group which it belongs (red is C; control, pink is Sf; sunflower).

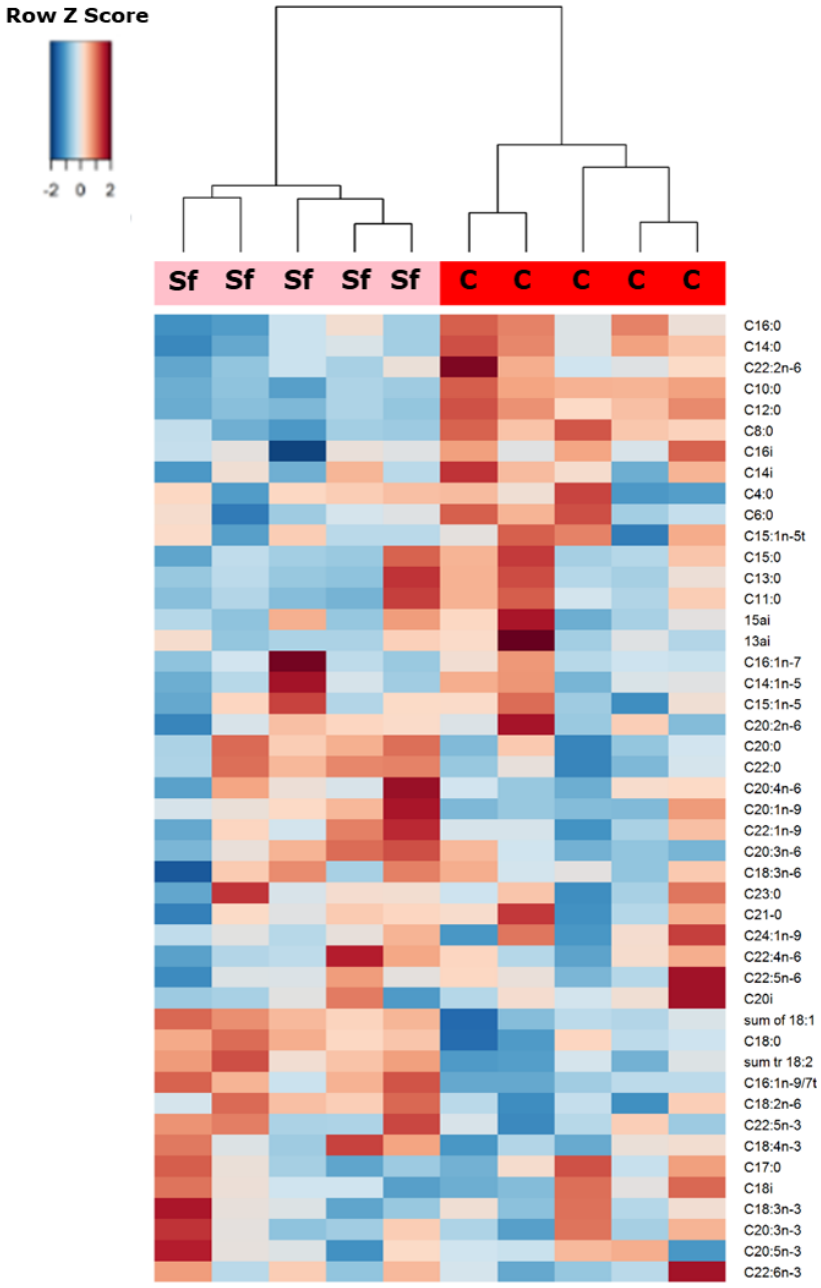



**Figure 3.11 Heat map for milk fatty acid profile at day 28 of lactation (control vs. sunflower).**

Samples from ewes fed a standard diet (control) or a standard diet + 3% sunflower oil (sunflower). Fatty acid content was transformed to a z-score. Fatty acid relative quantities are visualised by a colour scale ranges from bright red (increase) through to dark blue (decrease), see legend. Each rectangle represents an individual sample and is color-coded to indicate the specific dietary group which it belongs (red is C; control, pink is Sf; sunflower).

# Chapter 4. Effect of variations in milk fatty acid profile on body fat composition, organ weight, plasma metabolites and thermogenic capacity of brown adipose tissues in suckling lambs

## 4.1 Introduction

### 4.1.1 Overview

In Chapter 3, the effects of supplementing the maternal diet with canola or sunflower oil on milk fatty acid profiles were tested, and these changes were summarised in Table 3.12 (Section 3.6).

This chapter explores the effects of those changes in milk fatty acid profile on the offspring. The investigation includes the offsprings' body fat composition, plasma metabolites, organ weights and the potential thermogenic capacity in brown adipose tissue (BAT) depots.

### 4.1.2 Adipose tissue

Two main types of adipose tissue can be recognised in early life: those where the majority of the adipocytes are brown and those with completely white adipocytes. Both types are distributed in specific anatomical locations (Lotta et al., 2017; Symonds et al., 2015). Brown adipocytes are characterised by numerous mitochondria expressing *UCP1*, the most common marker of BAT (Cannon & Nedergaard, 2004). This protein, when activated, releases heat by the oxidation of glucose and free fatty acids (Fedorenko et al., 2012), which helps the newborn to maintain their body temperature. By the end of the first month of life, in sheep, BAT is gradually lost and replaced with WAT (Rockstroh et al., 2015; Symonds et al., 2015), which, unlike BAT, stores energy for later use. The cellular composition of WAT is influenced by endocrine factors (Symonds, 2013; Symonds et al., 2015). Moreover, individuals vary in their distribution of white adipocytes and the number of remaining brown adipocytes, which

can be an indicator of the risk of developing metabolic complications in later life (Bacha & Gidding, 2016; Virtanen et al., 2009).

In addition to the two main types of adipose tissue, there is a third type, known as 'beige' or 'BRITE' (BRown in whITE) - these are 'brown-like' adipocytes in WAT that are able to express *UCP1* when mice are exposed to cold (Xue et al., 2009) or by β3 adrenergic receptor stimulation (Himms-Hagen et al., 2000). BRITE adipocytes have 10% of the capacity of classic BAT as they possess much less *UCP1* (Nedergaard & Cannon, 2013) and are likely to be from a different lineage (Seale et al., 2008).

It is important to have a comprehensive understanding of the influence of the anatomical location of the adipose tissue and its developmental path (Lotta et al., 2017; Spalding et al., 2008) in order to shape successful dietary intervention. Unfortunately, although much research into fatty acid effects on BAT thermogenic capacity have been carried out (Calderon-Dominguez et al., 2016; Metges et al., 2003; Mollerup & Haugen, 1996; Pahlavani et al., 2017; Saha et al., 1998), these have not produced real progress in clarifying the functional differences between the adipose tissue depots throughout the body. This may be attributed to two main reasons. Firstly, most studies have been conducted in rodents (Calderon-Dominguez et al., 2016; Metges et al., 2003; Ohinata et al., 1998; Pahlavani et al., 2017; Saha et al., 1998) or in vitro (Mollerup & Haugen, 1996; Ohinata et al., 1998), not in larger mammals. Secondly, the majority of these studies have been carried out in an isolated manner without combining potentially influential factors, and therefore, they are not able to provide an overall view of BAT development and the differences between BAT depots (Gesta et al., 2007).

As seen in Chapter 3, most of the changes in milk fatty acid profile resulting from maternal fatty acid supplementation were in monounsaturated fatty acids (MUFAs) and n-6 polyunsaturated fatty acids (PUFAs). MUFA were increased in milk obtained from ewes supplemented with canola oil, and to lesser extent, with sunflower oil (Section 3.4.1.3); while n-6 PUFA were only increased with sunflower oil supplementation (Section 3.4.1.5). These findings were consistent with the fatty acid

composition of canola and sunflower oils as canola oil is higher in MUFA whilst sunflower oil is higher in n-6 PUFA (Section 3.1.3.1). Previous studies indicate that both maternal, and individual, supplementation with vegetable oil rich in PUFA modifies the fatty acid profile (Manso et al., 2011) and induces thermogenesis (Priego et al., 2013; Takeuchi et al., 1995) in suckling offspring of ewes and rats respectively. In most of such studies, supplementation commenced from late gestation and/or up to weaning (Manso et al, 2011; Ojha et al, 2015; Priego et al, 2013). However, in the present study, maternal supplementation commenced postpartum, giving the opportunity to investigate the effect of postpartum maternal fatty acid supplementation on the expression of genes involved in fatty acid oxidation, biosynthesis and storage in offspring adipose tissue depots.

In the present study, in order to obtain an overall and comparative overview into developmental changes within BAT depots in early life, three major adipose tissue depots (sternal, perirenal and epicardial) from young sheep, known to be populated with brown adipocytes in early postnatal life (Symonds et al., 2012), were studied and compared at day 7 and 28 of age. Particular emphasis was placed on comparing the expression profile of genes involved in MUFA and n-6 PUFA metabolism amongst these adipose tissue depots in order to have a deeper insight to differentiate between them. Further study of gene expression profiles of those major adipose tissue depots will be detailed in Chapter 5.

## 4.2 Hypotheses

As discussed in Chapter 3, Section 3.4.1.5, sunflower oil supplementation increased the milk concentration of linoleic acid (LA, C18:2 n-6) by day 28 of age. It has been reported that LA, and its derivative arachidonic acid (AA, C20:4 n-6), are associated with increased body fat (Javadi et al., 2004; Weiler, 2000) and promote pre-adipocyte differentiation (Massiera et al., 2003). Accordingly, I hypothesised that sunflower oil supplementation of maternal diet increased the fat mass and thus the total body weight of offspring because of an increase in milk LA concentration. This could be due to hypertrophy (an increase in cell size) and/or hyperplasia (an increase in cell numbers) as will discussed further in Chapter 6.

There was a modification in the fatty acid composition of maternal milk as a result of the supplemented maternal diet with fatty acid (results are summarised in Chapter 3, Table 3.12). It has been reported that dietary fatty acids can affect both the expression and activity of *UCP1* (Fernández-Quintela et al., 2010; Welter et al., 2016). Therefore, I hypothesised that supplementing the maternal diet with canola oil or sunflower oil during lactation could increase the expression of *UCP1* gene as a result of the reduction observed in the omega-6/omega-3 FA ratio and conjugated linoleic acid (CLA) in the milk.

Based on recent research from mice suggesting that the proportions of adipocyte progenitor cells derived from different lineages in individual adipose tissues varies dependent on the adipose tissue depot location (Calderon-Dominguez et al., 2016; Sanchez-Gurmaches et al., 2012) resulting in different expression profiles (Macotela et al., 2012; Rockstroh et al., 2015; Spalding et al., 2008), I hypothesised that different gene expression profiles would be apparent between the lambs' sternal, perirenal and epicardial adipose tissue depots.

## 4.3 Methods

The experimental model used in this chapter is summarised in Figure 4.1, and the methods used are described in detail in Chapter 2. As described in Chapter 3, all animal experimentation in this study was performed by Professor Michael Symonds, Dr. Mark Birtwistle, Dr. Viv Perry and Dr. Graeme Davies at the Sutton Bonington campus of the University of Nottingham.

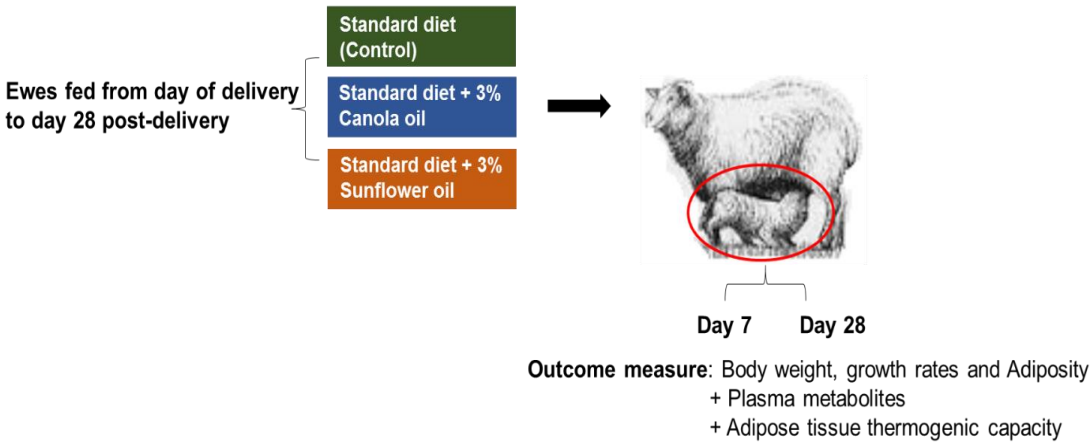



**Figure 4.1 Summary of experimental model used in Chapter 4.**
Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil. Offspring lambs were mother-fed from birth until day of tissue sampling. Day numbers refer to days of age. Within day of sampling, the mean of each outcome in each intervention group was compared to the corresponding control group by one-way ANOVA and Bonferroni post-hoc test unless otherwise stated.

Blood concentrations of triglycerides and cholesterol were measured to determine the change in blood lipids in response to the alteration of the milk fatty acid profile (Schaefer et al., 2016). Plasma glucose was measured to give an indication of whether altered milk composition effects glucose control (Blaak et al., 2012). Finally, plasma insulin-like growth factor 1 (IGF-1) and leptin were measured because they are associated with fat mass in early life (Bispham et al., 2003; Heasman et al., 1998). Professor Duane Keisler at the University of Missouri, USA, performed the IGF-1 and leptin analyses. All other plasma metabolite analyses were performed by Drs. Nigel

Kendall and Rachel Woods at the School of Veterinary Medicine, Sutton Bonington Campus, University of Nottingham.

The mRNA expression for some genes regulated in brown, white or BRITE adipose tissue was measured to evaluate the potential impact of altering the milk fatty acid profiles on the offspring. The total number of lambs included in the qPCR results varied among adipose tissue depots in both time points according to the availability of an adequate amount of tissue for RNA extraction especially from the epicardial adipose depot which is small in early life. Of the initial number of lambs included in this study, the results include data for 48 lambs for the sternal adipose tissue depot (Day 7: n=24; Day 28: n= 24), 47 for the perirenal adipose tissue depot (Day 7: n=22; Day 28: n= 25) and 27 for the epicardial adipose tissue depot (Day 7: n=12; Day 28: n= 15). The adjusted numbers for each group are shown in Figure 4.2.

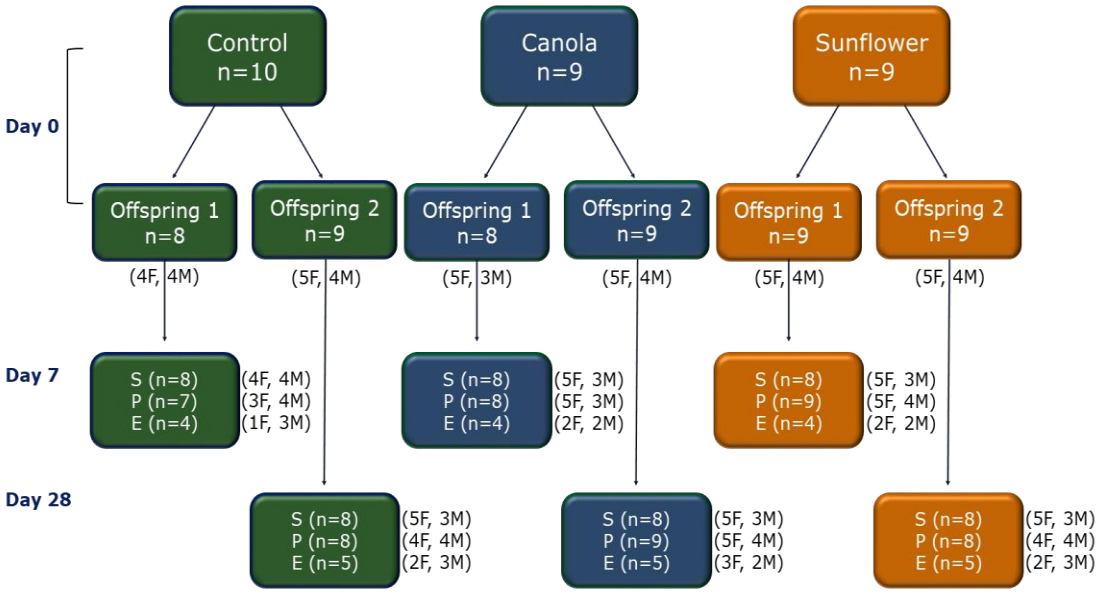



**Figure 4.2 Summary of qPCR experimental design.**

Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil. Offspring lambs were mother-fed from birth until day of tissue sampling. The day numbers indicate age in days. Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. Numbers of females (F) and males (M) were indicated under/beside each group.

### 4.3.1 Statistical analysis

For gene expression and plasma metabolites results, normalisation and data analysis were performed using free and open source packages from the Comprehensive R Archive Network (CRAN) project (http://cran.r-project.org/). The 'gcrma' function embedded in the 'affy' package was used for pre-processing data stages, including background correction, normalisation and probe match verification. In order to compare results among groups, the mean of outcome in each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test, and $q \leq 0.05$ was considered a significant result.

## 4.4 Results

### 4.4.1 Body weight

No significant differences were observed in body weight among the groups at birth, day 7 or day 28 in either gender (Figure 4.3). Growth rates were separately calculated for birth to day 7 and birth to day 28 for females and males. In the sunflower oil supplementation group, females showed a more rapid growth rate for birth to day 7 than controls ($q$=0.04; Figure 4.4) and there was a trend to more rapid growth from birth to day 28, compared to the female controls although this did not reach statistical significance ($q$=0.059).

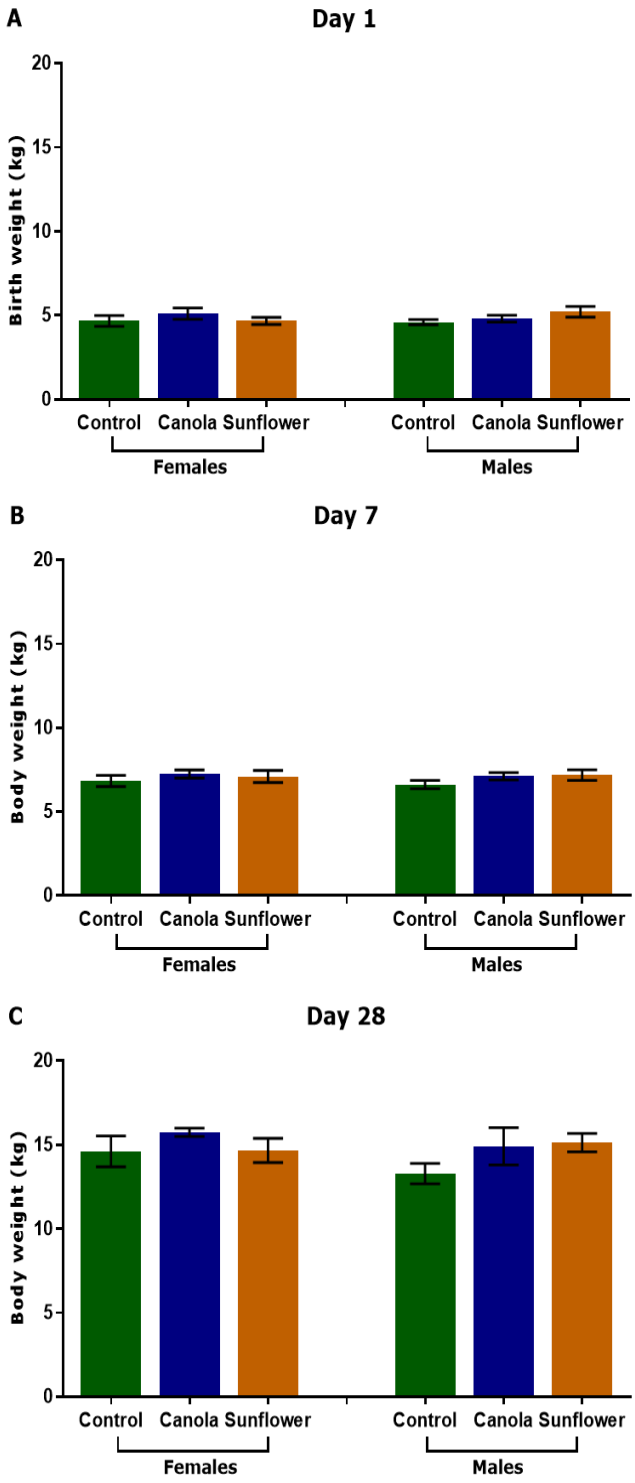



**Figure 4.3 Lamb body weight (kg) by dietary group and gender at (A) day 1, (B) day 7 and (C) day 28.**

The dietary group relates to maternal diets: control (standard diet, green), canola (standard diet +3% canola oil, blue) and sunflower (standard diet +3% sunflower oil, orange). At day 7, females: control, n=9; canola, n=8; sunflower, n=10; males: control, n=7; canola, n=7; sunflower, n=8. At day 28, 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 one-way ANOVA and Bonferroni post-hoc test.

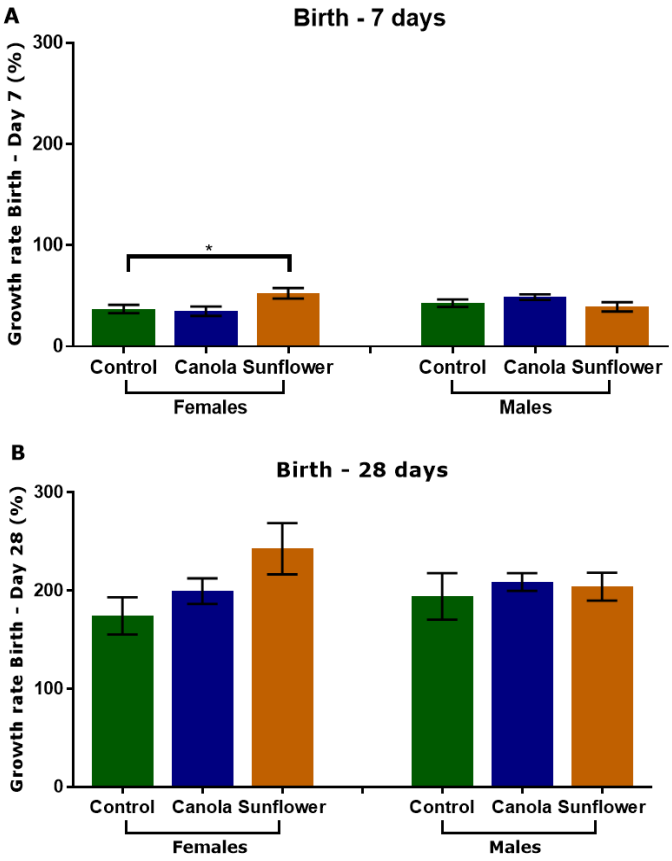



**Figure 4.4 Lamb growth rates (percentage increase in weight) by dietary group and gender (A) for birth to day 7 and (B) birth to day 28.**

The dietary group relates to maternal diets: control (standard diet, green), canola (standard diet +3% canola oil, blue) and sunflower (standard diet +3% sunflower oil, orange). At day 7, females: control, n=9; canola, n=8; sunflower, n=10; males: control, n=7; canola, n=7; sunflower, n=8. At day 28, 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 one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by * $q$=0.04.

### 4.4.2 Adipose tissue weights

Omental, pericardial, perirenal and sternal adipose tissues, weighed at days 7 and 28 of age, and depot weight, relative to body weight (g/kg of body weight), are shown in Figures 4.5 and 4.6, respectively.

At day 7, there were no changes in the weight of the adipose tissue depots, relative to body weight, between dietary groups in either gender.

At day 28, significant increases were seen in the omental (17%, $q$=0.02), pericardial (53%, $q$=0.03) and perirenal (76%, $q$=0.01) adipose tissue depot weights among

female lambs in the sunflower oil supplemented group. In the canola oil supplemented group, an increase of 47% was observed in the females' sternal adipose tissue relative to body weight ($q$=0.04). No changes were observed in the males between the dietary groups.

The combined sum of the adipose tissue depot weights was calculated. At day 7, there were no differences between dietary groups in either gender (Figure 4.5). At day 28, combined adipose tissue weight in the females of both the canola and sunflower groups was higher than that of the control group by 29% and 49% respectively ($q$=0.04 and $q$=0.003) (Figure 4.6). No effects of fatty acid supplementation were seen in the combined adipose tissue depot weights of male lambs.

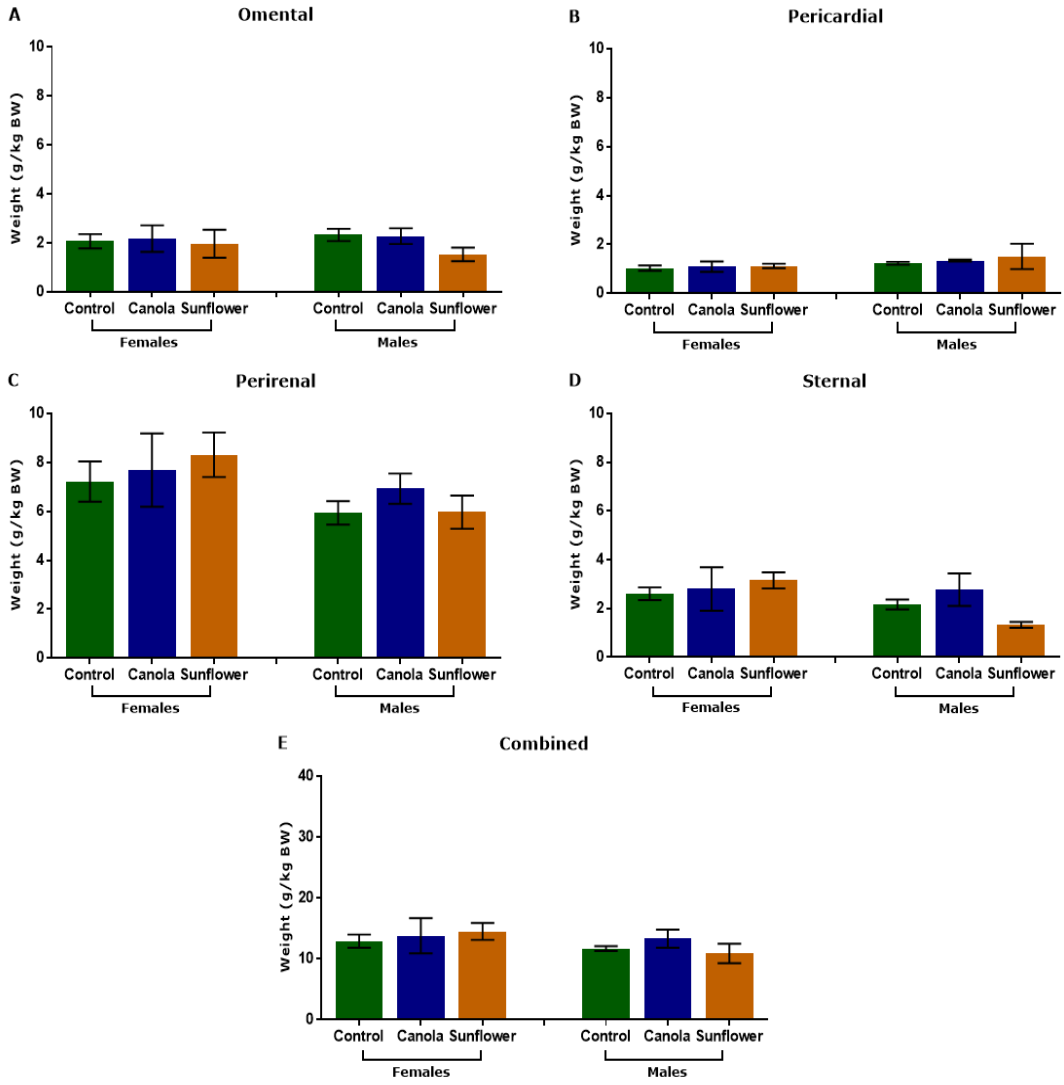



**Figure 4.5 Lamb (A) omental, (B) pericardial, (C) perirenal, (D) sternal and (E) combined adipose tissue depots weight to body weight (g/kg) at day 7 of age by dietary group and gender.**

Adipose tissue depots were taken from the offspring of ewes fed a standard diet (Control: 4 females and 4 males, green), a standard diet + 3% canola oil (Canola: 4 females and 3 males, blue) or a standard diet + 3% sunflower oil (Sunflower: 5 females and 4 males, orange). The combined figure is the total adiopse tissue from the four adipose tissue depots weighed. Values are mean ± SEM. Within each individual day's sampling, the mean of each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test.

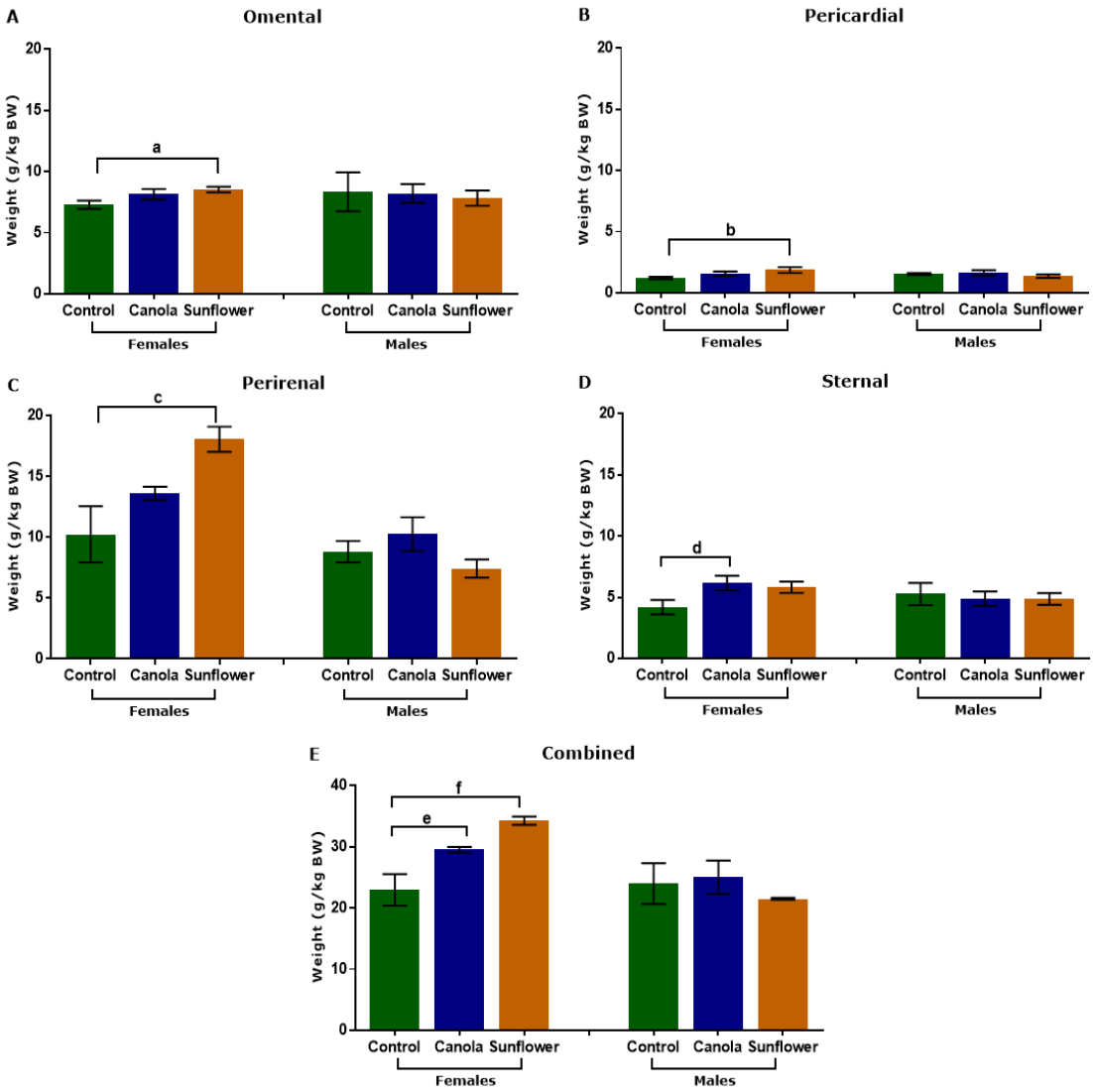



**Figure 4.6 Lamb (A) omental, (B) pericardial, (C) perirenal, (D) sternal and (E) combined adipose tissue depots weight relative to body weight (g/kg) at day 28 of age by dietary group and gender.**

Adipose tissue depots were taken from the offspring of ewes fed a standard diet (Control: 4 females and 4 males, green), a standard diet + 3% canola oil (Canola: 4 females and 3 males, blue) or a standard diet + 3% sunflower oil (Sunflower: 5 females and 4 males, orange). The combined figure is the total adipose tissue from the four adipose tissue depots weighed. Values are mean ± SEM. Within each individual day's sampling, the mean of each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test (a: $q$=0.02; b: $q$=0.03; c: $q$=0.01; d: $q$=0.04; e: $q$=0.04; f: $q$=0.003).

Lambs' adipose tissue depots weight to body weight (g/kg) change from day 7 to day 28 of age by dietary group and gender are shown in Table 4.1. Results showed gender variations cross all dietary groups. In controls, there was no effect of age on female lambs' adipose tissue depots weight except in omental ($q$>0.0001) while omental and sternal showed an increase with age in males ($q$=0.0002 and $q$=0.004) respectively.

In canola group, all studied adipose depots exhibited increase with age except the pericardial in females but only omental showed the same in males ($q$=0.0005). In sunflower group, a significant increase with age was seen in all depots in females and in omental and sternal in males.

**Table 4.1 change in lamb adipose tissue depots weight to body weight (g/kg) from day 7 to day 28 of age by dietary group and gender.**

| | Females | | | Males | | |
|---|---|---|---|---|---|---|
| | **Control (*n*=9) 7D=4, 28D=5** | **Canola (*n*=9) 7D=4, 28D=5** | **Sunflower (*n*=10) 7D=5, 28D=5** | **Control (*n*=8) 7D=4, 28D=4** | **Canola (*n*=7) 7D=3, 28D=4** | **Sunflower (*n*=8) 7D=4, 28D=4** |
| Omental | ↑ $q$>0.0001 | ↑ $q$>0.0001 | ↑ $q$>0.0001 | ↑ $q$>0.0001 | ↑ $q$>0.0001 | ↑ $q$>0.0001 |
| Pericardial | NS | NS | ↑ $q$=0.009 | NS | NS | NS |
| Perirenal | NS | ↑ $q$=0.012 | ↑ $q$>0.0001 | NS | NS | NS |
| Sternal | NS | ↑ $q$=0.001 | ↑ $q$=0.005 | ↑ $q$>0.004 | NS | ↑ $q$=0.002 |

Adipose tissue depots were taken from the offspring of ewes fed a standard diet (Control: 4 females at day 7 and 5 at day 28; 4 males at day 7 and 4 at day 28, green), a standard diet + 3% canola oil (Canola: 4 females at day 7 and 5 at day 28; 3 males at day 7 and 4 at day 28, blue) or a standard diet + 3% sunflower oil (Sunflower: 5 females at day 7 and 5 at day 28; 4 males at day 7 and 4 at day 28, orange). Within each gender, the adipose tissue weight of individuals in each intervention group was compared between sampling age by one-way ANOVA and Bonferroni post-hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$).

### 4.4.3 Organ weight

Weighed organs included: adrenal, brain, heart, kidneys, liver, lungs, pancreas, spleen, lymph nodes, thymus and thyroid. No changes in organ weight as a result of a maternal dietary supplementation with canola or sunflower oil were seen at day 7 (Table 4.2). At day 28, few changes were observed in the organ weights of males, but not females (Table 4.3). In the canola oil supplemented group, male lambs showed a 29% ($q$=0.006) decrease in lymph nodes weight, relative to body weight. In the sunflower oil supplemented group, decreases of 15% ($q$=0.047) and 10% ($q$= 0.04) respectively, were observed in the relative weights of brain and heart, in males when compared to controls. These reductions in relative organ weight may be because other organ(s) (e.g. lungs) growing disproportionately more or indicate an overall slower growth rate rather than an actual decrease in organ weight.

**Table 4.2 Lamb organ weight relative to body weight (g/kg) at day 7 of age by dietary group and gender.**

| Organ | Females | | | Effect (% change) | Males | | | Effect (% change) |
|---|---|---|---|---|---|---|---|---|
| | Control (n=4) | Canola (n=4) | Sunflower (n=5) | | Control (n=4) | Canola (n=3) | Sunflower (n=4) | |
| Adrenal | 0.08±0.01 | 0.08±0.01 | 0.09±0.01 | NS | 0.09±0.001 | 0.08±0.01 | 0.08±0.01 | NS |
| Brain | 7.25±0.36 | 7.07±0.35 | 6.49±0.60 | NS | 7.32±0.26 | 7.10±0.31 | 7.18±0.65 | NS |
| Heart | 7.70±0.29 | 7.69±0.32 | 8.21±0.47 | NS | 7.57±0.13 | 7.53±0.52 | 7.77±0.30 | NS |
| Kidneys | 6.99±0.30 | 6.39±0.40 | 6.26±0.24 | NS | 6.99±0.10 | 6.86±0.35 | 6.89±0.72 | NS |
| Liver | 25.47±1.68 | 23.02±1.14 | 25.77±0.81 | NS | 27.67±0.50 | 27.22±0.76 | 25.72±1.12 | NS |
| Lungs | 23.15±0.84 | 26.35±2.35 | 20.50±1.02 | NS | 21.40±1.15 | 23.37±1.80 | 21.92±0.38 | NS |
| Pancreas | 0.72±0.04 | 0.67±0.05 | 0.81±0.18 | NS | 0.67±0.10 | 0.74±0.05 | 0.89±0.03 | NS |
| Spleen | 2.90±0.41 | 2.78±0.31 | 2.67±0.44 | NS | 3.15±0.21 | 3.45±0.46 | 1.94±0.96 | NS |
| Lymph node | 0.51±0.08 | 0.49±0.05 | 0.45±0.05 | NS | 0.57±0.04 | 0.49±0.02 | 0.50±0.09 | NS |
| Thymus | 1.46±0.30 | 1.20±0.08 | 1.62±0.11 | NS | 1.07±0.11 | 1.19±0.26 | 1.37±0.48 | NS |
| Thyroid | 0.10±0.02 | 0.05±0.01 | 0.08±0.01 | NS | 0.08±0.01 | 0.07±0.01 | 0.13±0.03 | NS |

Organs were taken from the offspring of ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) or a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within each individual day's sampling, the mean of each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test. NS = not significant ($q \geq 0.05$).

**Table 4.3 Lamb organ weight relative to body weight (g/kg) at day 28 of age by dietary group and gender.**

| Organ | Females | | | Effect (% change) | Males | | | Effect (% change) |
|---|---|---|---|---|---|---|---|---|
| | Control (n=4) | Canola (n=4) | Sunflower (n=5) | | Control (n=4) | Canola (n=4) | Sunflower (n=4) | |
| Adrenal | 0.06±0.005 | 0.05±0.004 | 0.06±0.004 | NS | 0.05±0.01 | 0.05±0.002 | 0.06±0.01 | NS |
| Brain | 4.30±0.39 | 3.39±0.15 | 4.34±0.25 | NS | 4.24±0.19 | 4.21±0.30 | 3.61±0.17 * | *↓%15 $q$=0.047 |
| Heart | 6.38±0.26 | 6.53±0.35 | 6.56±0.18 | NS | 6.96±0.11 | 6.23±0.24 | 6.86±0.54 * | *↓%10 $q$=0.04 |
| Kidneys | 5.10±0.20 | 5.20±0.03 | 5.41±0.23 | NS | 5.38±0.15 | 5.62±0.43 | 5.34±0.12 | NS |
| Liver | 19.82±1.24 | 22.25±1.23 | 23.08±0.84 | NS | 23.59±0.55 | 22.92±0.89 | 23.29±1.21 | NS |
| Lungs | 18.00±1.25 | 15.70±0.98 | 16.95±0.67 | NS | 16.00±0.50 | 16.23±0.37 | 17.76±1.10 | NS |
| Pancreas | 0.63±0.14 | 0.63±0.07 | 0.78±0.18 | NS | 0.62±0.07 | 0.76±0.15 | 0.62±0.09 | NS |
| Spleen | 4.46±0.66 | 4.55±0.63 | 3.85±0.64 | NS | 4.09±0.64 | 4.37±0.64 | 4.14±0.51 | NS |
| Lymph node | 0.57±0.09 | 0.48±0.05 | 0.44±0.04 | NS | 0.51±0.02 | 0.36±0.03** | 0.44±0.03 | **↓%29 $q$=0.006 |
| Thymus | 2.60±0.27 | 2.64±0.47 | 2.66±0.10 | NS | 2.50±0.22 | 2.30±0.15 | 2.70±0.27 | NS |
| Thyroid | 0.05±0.01 | 0.05±0.01 | 0.06±0.01 | NS | 0.06±0.01 | 0.05±0.003 | 0.05±0.01 | NS |

Organs were taken from the offspring of ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) or a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within each individual day's sampling, the mean of each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test. Directional arrows denote significant differences. NS = not significant ($q \geq 0.05$).

### 4.4.4 Plasma metabolites

Plasma concentrations of triglycerides, glucose, cholesterol, leptin and IGF-1 were measured at days 7 and 28, and the results are shown in Tables 4.4 and 4.5, respectively.

No effects of fatty acid supplementation were seen in the lambs' plasma IGF-1 concentrations.

Plasma cholesterol (mmol/l) concentrations were not affected by dietary intervention in either gender at day 7. By day 28, plasma cholesterol concentrations in female lambs were significantly lower in both the canola and sunflower groups, respectively, compared with the controls (Day 28: Canola group: 25% ($q$=0.01); Sunflower group: 19% ($q$=0.03)). No changes were observed in cholesterol concentrations among males at day 28.

At day 7, plasma glucose concentrations were 13% higher in males in the sunflower group compared with the control group ($q$=0.01), although this difference was not observed in females. In contrast, although plasma glucose concentrations in male offspring were not affected at day 28, at the same time point they were 16% higher in female offspring compared with the control group ($q$=0.003). In the canola group, no significant changes were observed in the plasma glucose concentrations on either day 7 or 28.

Plasma leptin concentrations were higher than those of the control group by 26% in the canola oil supplemented females ($q$=0.002) and by 41% in the sunflower oil supplemented females ($q$=0.01) at day 7. At day 28, plasma leptin concentrations were 80% higher in female lambs exposed to maternal canola oil supplementation and in 62% in those exposed to maternal sunflower oil supplementation (both $q$=0.03) compared with the control group. There were no changes in plasma leptin concentrations among males in any of the dietary groups at either time points.

Plasma triglyceride concentrations in the sunflower group were 38% more than those of the control group ($q$=0.01) among females at day 7 with no differences were observed among males at day 7 or either genders at day 28. In the canola group, there were no differences in triglyceride concentrations compared with the control group for either genders at days 7 or 28.

**Table 4.4 Plasma metabolite values at day 7 of age by dietary group and gender.**

| Plasma metabolite | Females | | | Effect (% change) | Males | | | Effect (% change) |
|---|---|---|---|---|---|---|---|---|
| | Control (n=4) | Canola (n=4) | Sunflower (n=5) | | Control (n=4) | Canola (n=3) | Sunflower (n=3) | |
| Triglycerides (mmol/l) | 0.55±0.02 | 0.67±0.07 | 0.76±0.05** | 38%↑ $q$=0.01 | 0.51±0.17 | 0.80±0.30 | 0.75±0.18 | NS |
| Glucose (mmol/l) | 7.04±0.67 | 6.67±0.17 | 6.32±0.42 | NS | 6.41±0.17 | 6.03±1.29 | 7.25±0.04** | 13%↑ $q$=0.01 |
| Cholesterol (mmol/l) | 2.11±0.07 | 2.19±0.12 | 1.77±0.12 | NS | 1.85±0.15 | 1.71±0.43 | 2.20±0.24 | NS |
| Leptin (ng ml−1) | 2.36±0.08 | 2.97±0.18* | 3.32±0.23** | 26%↑ $q$=0.002<br>41% ↑$q$=0.01 | 2.79±0.36 | 2.89±0.32 | 2.54±0.16 | NS |
| IGF-1 (ng ml−1) | 126±5 | 100±17 | 137±16 | NS | 150±10 | 169±2 | 158±9 | NS |

Blood samples were taken from the offspring of ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) or a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within each individual day's sampling, the mean of each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test. Directional arrows denote significant differences. NS = not significant ($q$≥0.05).

**Table 4.5 Plasma metabolite values at day 28 of age by dietary group and gender.**

| Plasma metabolite | Females | | | Effect (% change) | Males | | | Effect (% change) |
|---|---|---|---|---|---|---|---|---|
| | Control (n=5) | Canola (n=5) | Sunflower (n=5) | | Control (n=4) | Canola (n=5) | Sunflower (n=5) | |
| Triglycerides (mmol/l) | 0.76±0.16 | 0.71±0.17 | 0.70±0.07 | NS | 0.85±0.05 | 0.83±0.03 | 1.03±0.28 | NS |
| Glucose (mmol/l) | 6.79±0.17 | 6.98±0.14 | 7.88±0.20 ** | 16%↑ $q$=0.003 | 8.42±0.50 | 7.72±0.19 | 7.55±0.37 | NS |
| Cholesterol (mmol/l) | 5.46±0.32 | 4.12±0.18 * | 4.44±0.23 ** | 25%↓ $q$=0.01<br>19%↓ $q$=0.03 | 4.06±0.42 | 4.68±0.59 | 4.00±0.53 | NS |
| Leptin (ng ml−1) | 2.64±0.20 | 4.74±0.77* | 4.28±0.60* | 80%↑ $q$=0.03<br>62%↑ $q$=0.03 | 3.04±0.42 | 3.04±0.42 | 3.04±0.42 | NS |
| IGF-1 (ng ml−1) | 186±12 | 228±20 | 221±31 | NS | 319±42 | 254±34 | 273±9 | NS |

Blood samples taken from the offspring of ewes fed a standard diet (Control), a standard diet + 3% canola oil (Canola) or a standard diet + 3% sunflower oil (Sunflower). Values are mean ± SEM. Within each individual day's sampling, the mean of each intervention group was compared with the control group by one-way ANOVA and Bonferroni post-hoc test. Directional arrows denote significant differences. NS = not significant ($q$≥0.05).

### 4.4.5 The effect of supplementing maternal diet with fatty acids on gene expression in offspring adipose tissue in the first month

The mRNA expression of genes of interest was tested in three major adipose tissue depots (sternal, perirenal and epicardial) of lambs at days 7 and 28. As no differences were observed in the gene-expression results between females and males, all gene-expression results were combined.

To facilitate interpretation of the results, genes of interest were classified into four main groups: (1) BAT - thermogenic genes, (2) WAT - adipogenic genes, (3) BRITE - development genes and (4) fatty acid metabolism genes.

#### 4.4.5.1 BAT/thermogenic genes

The relative mRNA expression in adipose tissue of *UCP1* and *PRDM16*, two genes generally used as BAT markers (Pope et al., 2014), were tested to examine the effect of fatty acid supplementation on BAT - thermogenic gene expression at days 7 and 28 and on the expression pattern between adipose tissue depots. The results are shown in Figure 4.7.

There were no visible effects of maternal dietary supplementation with canola or sunflower oils on gene expression of *UCP1* in the sternal, perirenal or epicardial depots on day 7 and 28 compared with controls. When the data from the three adipose tissue depots was compared at 7 days of age, the mean mRNA relative expression of *UCP1* was significantly higher in the epicardial adipose tissue depot than in the sternal in all dietary groups. Moreover, the mean *UCP1* gene expression was still higher in the epicardial than in the sternal adipose tissue depot in both the control (8%, $q$=0.006) and sunflower-oil-supplemented groups (25%, $q$=0.0001). At day 28, no significant differences were found between adipose tissue depots in the canola-oil-supplemented group. However, it was higher than that in the perirenal adipose tissue depot, irrespective of maternal diet (c.50%, $q$<0.0001). . No differences were observed between the perirenal and sternal adipose tissue depots in any dietary group.

Variation in the percentage of difference of *UCP1* mRNA relative expression between the epicardial and sternal adipose tissue depots across dietary groups could indicate the differential effect of fatty acid supplementation on the thermogenic capacity of adipose tissue depots.

The relative expression of *PRDM16* mRNA was stable across dietary groups in each of the three adipose depots at both time points. In each dietary group,the highest relative expression at 7 days of age was found in the epicardial adipose tissue depot (c.30% more than the sternal and c.15% more than the perirenal), followed by the perirenal (c.40% more than sternal) and then the sternal ($q$<0.0001) depots. At day 28, no differences were seen between the epicardial and the perirenal adipose tissue depots; however, they were 20% higher than in the sternal adipose tissue depots ($q$<0.0001).

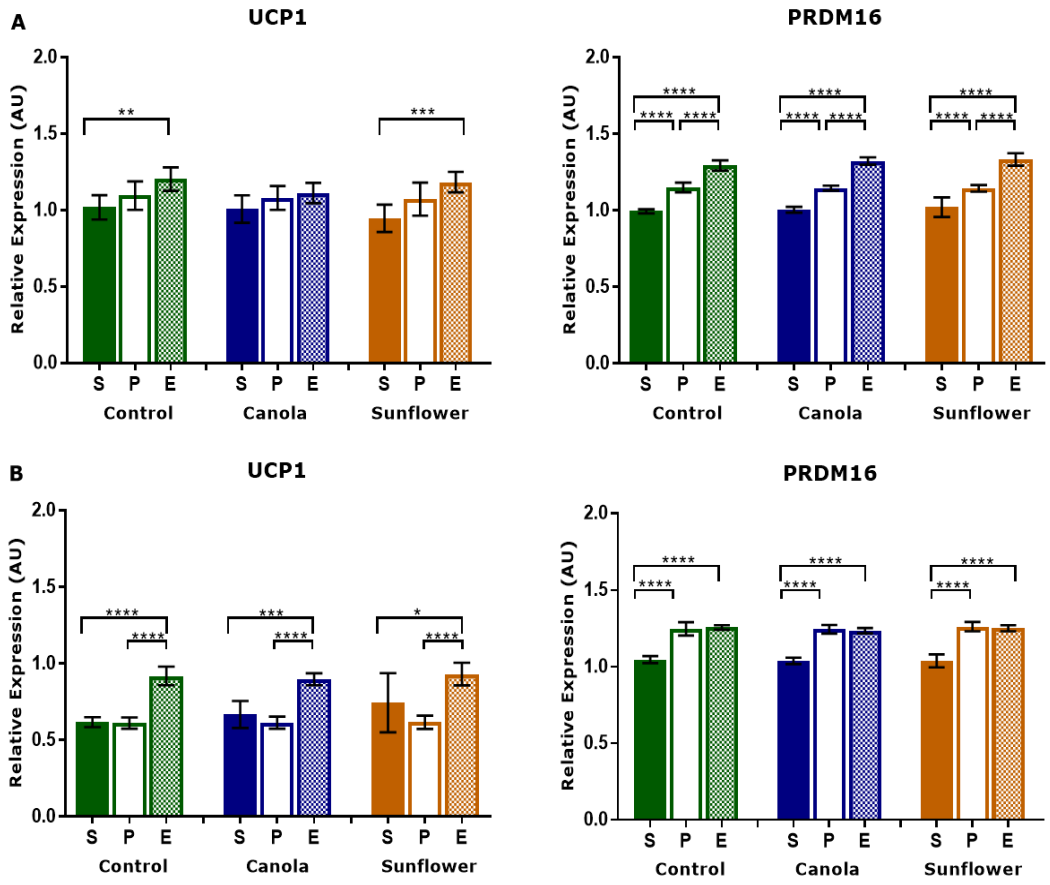



**Figure 4.7 Relative mRNA expression of BAT/thermogenic genes in major adipose tissue depots at (A) day 7 and (B) day 28 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group, and depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$q \leq 0.05$; **$q \leq 0.01$; ***$q \leq 0.001$ and ****$q \leq 0.0001$.

### 4.4.5.2 WAT genes

The relative mRNA expression of *leptin*, the most distinctive WAT gene (Pope et al., 2014; Walden et al., 2012), was examined in offspring sternal, perirenal and epicardial adipose tissues at days 7 and 28. The results are shown in Figure 4.8. At both time points, the relative expressions of *leptin* were not affected by maternal fatty acid supplementation in all adipose tissue depots.

On day 7, the epicardial adipose tissue depot was the site of the highest mRNA expression of *leptin* ($q$<0.0001) compared with the sternal and perirenal adipose tissue depots across all dietary groups. There were no differences in this regard between the sternal and perirenal adipose tissue depots. However, by day 28, the perirenal adipose tissue depot exhibited significantly greater *leptin* mRNA expression than the sternal depot (by 20%, $q$<0.0001), although this remained lower than that in the epicardial in all dietary groups.

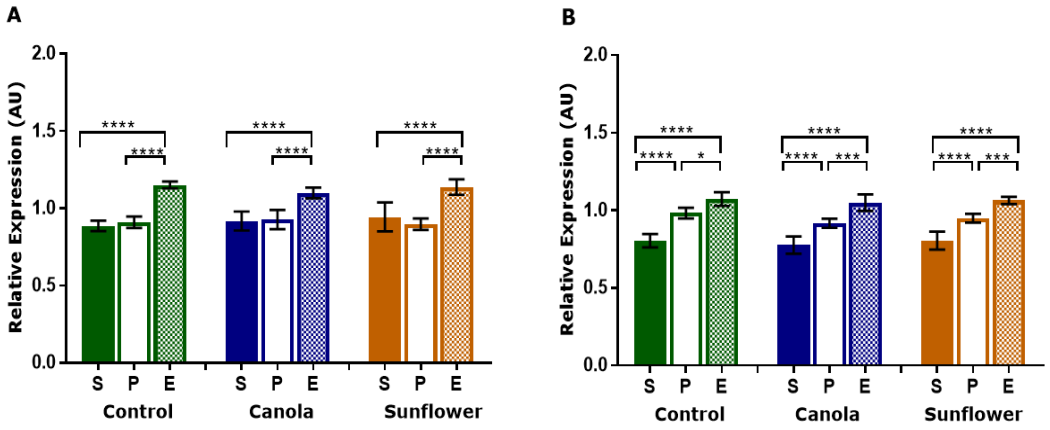



**Figure 4.8 Relative mRNA expression of leptin, WAT gene, in major adipose tissue depots at (A) day 7 and (B) day 28 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group, and the depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$q$≤0.05; ***$q$≤0.001 and ****$q$≤0.0001.

#### 4.4.5.3 BRITE/development genes

The mRNA relative expression of developmental genes *HOXC9* and *SHOX2* (Gesta et al., 2006; Yamamoto et al., 2010) at days 7 and 28 in the offsprings' major adipose tissue depots are shown in Figure 4.9. No variations in relative expression were seen between the supplemented groups and the control group at either time point.

The pattern of mRNA relative expression of *HOXC9* and *SHOX2* varied across the three adipose tissue depots between days 7 and 28. At day 7, mRNA expression in the epicardial adipose tissue depot was the highest, followed by the sternal and then the perirenal adipose tissue depots in all dietary groups. No differences were seen in *HOXC9* mRNA expression between the control and supplemented groups in the individual adipose tissue depots. However, the percentage of difference in the expression between the epicardial and sternal adipose tissue depots increased from 47% in the control group to 57% in the supplemented groups ($q$<0.0001) and from 71% to c.85% between the epicardial and perirenal ($q$<0.0001) adipose tissue depots. At the same time point, the expression was higher in the sternal, than in the perirenal, adipose tissue depot by c.18% in all dietary groups ($q$<0.01). For *SHOX2*, the percentages of difference in the expression among adipose tissue depots were low and consistent across dietary groups. By day 28, the perirenal adipose tissue depot switched from being that with lowest expression of developmental genes to be the highest one with more variation seen in *HOXC9* and *SHOX2* mRNA relative expressions across adipose tissue depots. The mRNA relative expression of *HOXC9* was higher in the perirenal, than in the sternal and epicardial adipose tissue, depot by c.10% ($q$<0.01), regardless of dietary group, while no differences were seen in the expression of *SHOX2* between the perirenal and epicardial adipose tissue depots, which were both higher than the sternal depot by c.30% ($q$<0.0001).

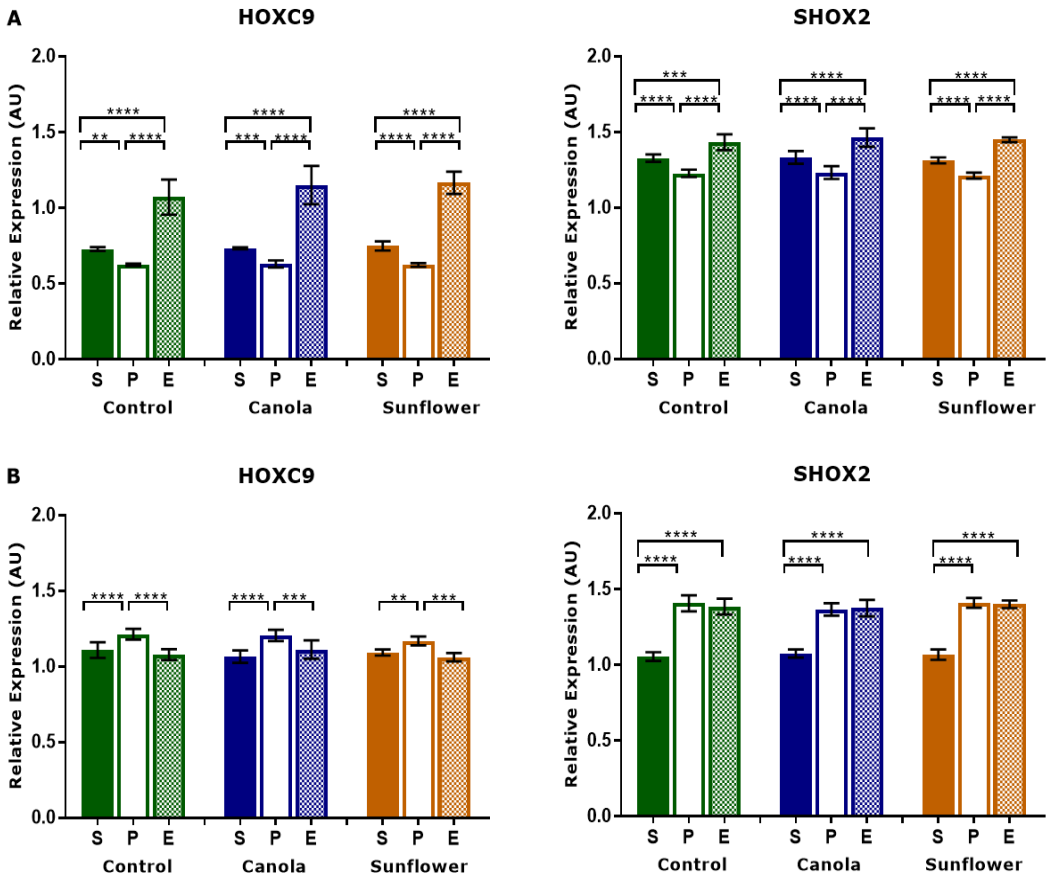



**Figure 4.9 Relative mRNA expression of BRITE/development genes in major adipose tissue at (A) day 7 and (B) day 28 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal, and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group and depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by $**q \leq 0.01$; $***q \leq 0.001$ and $****q \leq 0.0001$.

#### 4.4.5.4 Fatty acid metabolism genes

A set of genes associated with fatty acid metabolism, including β oxidation, fatty acid synthesis and storage were examined in offspring adipose tissue depots to provide an insight into the effects of maternal fatty acid supplementation, acting through maternal milk, during the early life of sheep. Genes examined in this section were classified into two groups: genes involved in fatty acid oxidation, namely *ACOT11*, *RIP140*, *COX1* and *COX2*; and genes involved in fatty acid synthesis and storage, namely *FADS2*, *FAS*, *SCD1*, *PPARγ* and *FABP3* (see Figures 4.10-13).

#### 4.4.5.4.1 Genes involved in fatty acid oxidation

At both 7 and 28 days, no significant differences were seen in the relative expression of these genes between the supplemented groups and controls in any depot (Figures 4.10-11).

Maternal fatty acid supplementation with either canola or sunflower oil exhibited differential effects between depots and stage of development. The epicardial depot showed the highest relative expression across all dietary groups in both time points ($q$<0.0001). On day 7, no differences in relative gene expression were seen between the sternal and perirenal depots, except for *ACOT11*, which was higher in the perirenal adipose tissue depot in all dietary groups ($q$<0.01) and for *RIP140* in the sternal, only in controls ($q$=0.047) (Figure 4.10). By day 28, the relative expression of all tested genes was significantly higher in the perirenal, than in the sternal, adipose tissue depot ($q$<0.0001), with no differences seen between the epicardial and perirenal for *ACOT11* in the dietary groups and for *COX2* in the sunflower oil supplemented group (Figure 4.11).

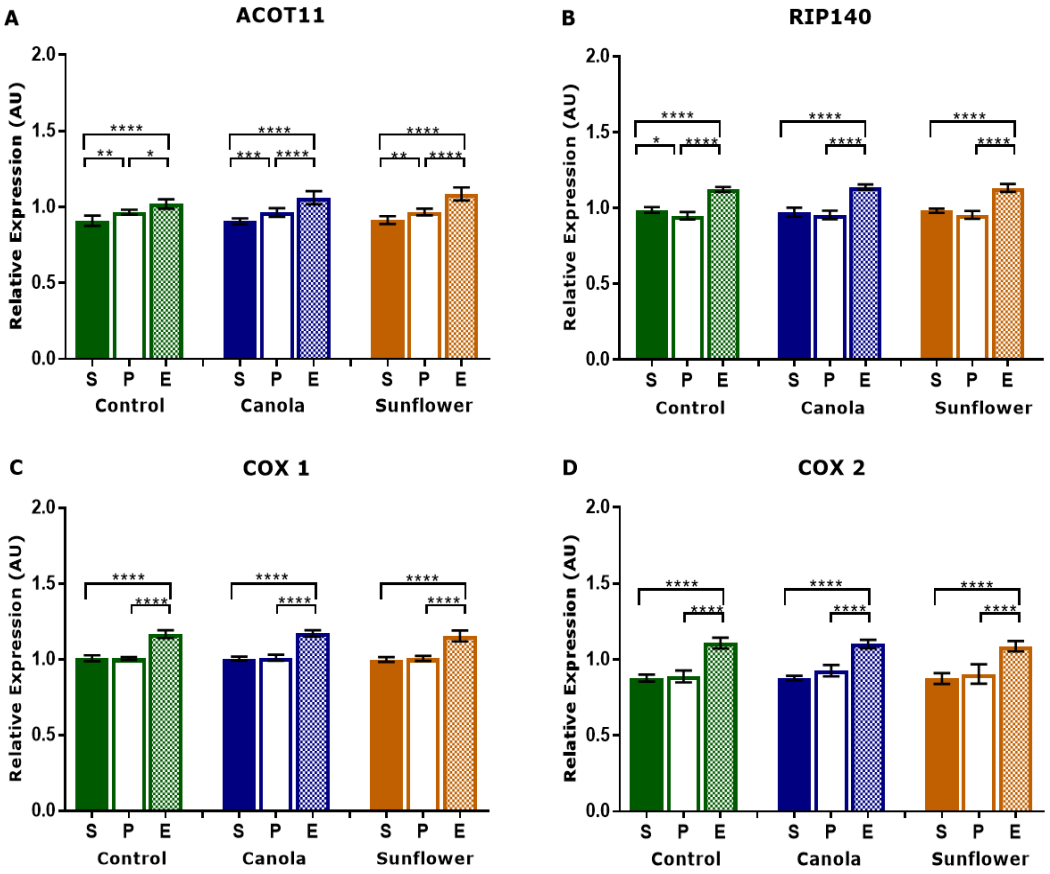



**Figure 4.10 Relative mRNA expression of genes (A) *ACOT11*, (B) *RIP140*, (C) *COX1* and (D) *COX2* which involved in fatty-acid oxidation in major adipose tissue on day 7 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group, and depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$q \leq 0.05$; **$q \leq 0.01$; ***$q \leq 0.001$ and ****$q \leq 0.0001$.

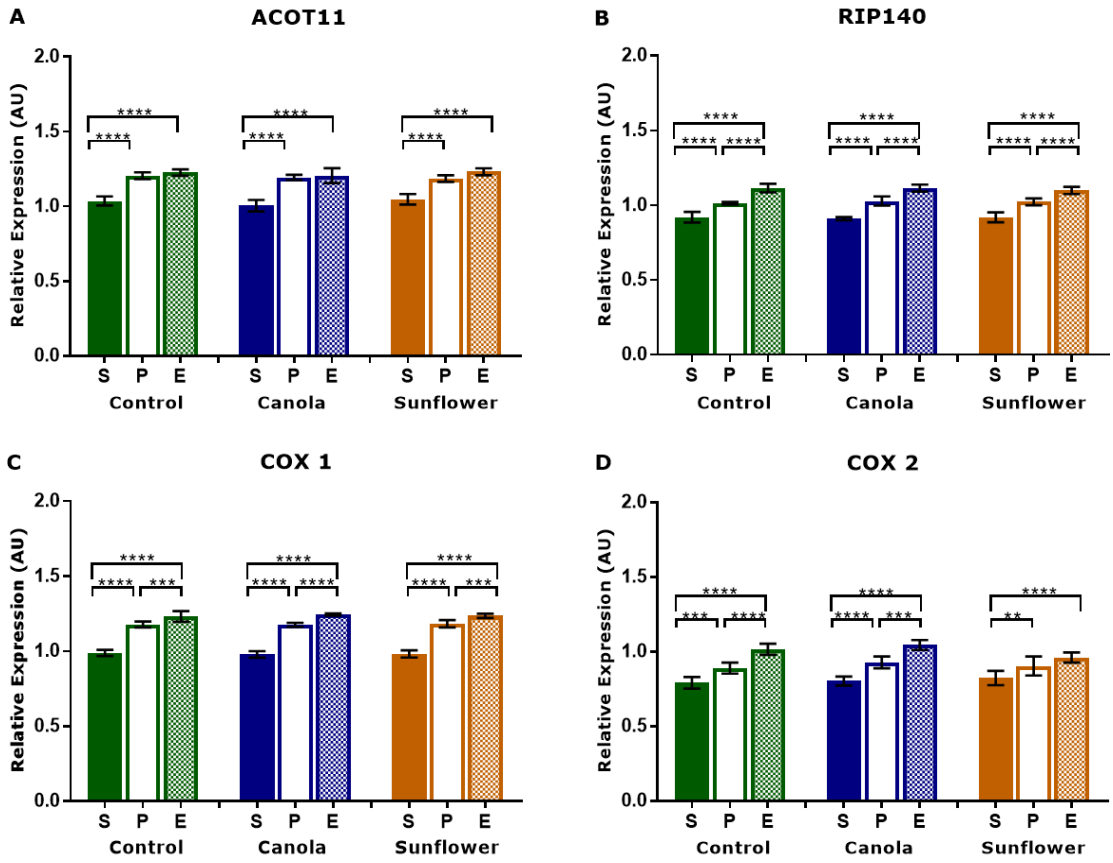



**Figure 4.11 Relative mRNA expression of genes (A) *ACOT11*, (B) *RIP140*, (C) *COX1* and (D) *COX2* which involved in fatty-acid oxidation in major adipose tissue on day 28 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group, and depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by **$q \leq 0.01$; ***$q \leq 0.001$ and ****$q \leq 0.0001$.

#### 4.4.5.4.2 Genes involved in fatty acid synthesis, storage and transfer

Sternal, perirenal and epicardial adipose tissue mRNA relative expression of genes involved in fatty acid synthesis, storage and transfer (*FADS2*, *FAS*, *SCD1*, *PPARγ* and FABP3) were examined at days 7 and 28, and shown in Figures 4.12 and 4.13 respectively.

Maternal fatty supplementation with sunflower oil resulted in an increase in the mRNA relative expressions of *FADS2* in the epicardial adipose tissue depot compared with the

controls ($q$=0.021) at day 7. However, by day 28, no significant differences were seen between the supplemented groups and controls in the expressions of these genes in any depot.

Comparing the mRNA relative expression of genes involved in fatty-acid synthesis and storage shows different patterns between these genes across adipose tissue depots that were influenced by maternal supplementation. At day 7, the pattern of gene expression was the same for *FADS2* and PPARγ, with the epicardial adipose tissue depot being the site of the highest expression, followed by the perirenal, then the sternal ($q$<0.0001); with the latter significantly lower than the perirenal ($q$<0.05) in the supplemented groups but not controls. The *FAS* and *SCD1* genes shared the same pattern of gene expression, with no differences seen between the sternal and perirenal adipose tissue depots, in both of which expression was lower than that in epicardial fat ($p$<0.0001) in all dietary groups. Similarly, gene expression of FABP3 was higher in the epicardial adipose tissue depot than in the sternal (q<0.0001) and in the perirenal (q≤0.010.01) within supplemented groups but not control (Figure 4.12). By day 28, gene expression of *FADS2*, *SCD1* and *PPARγ* was the lowest in the sternal then perirenal then epicardial ($q$<0.0001). Moreover, a similar pattern was seen in *FAS* and, to a lesser extent, in *FABP3* with no differences between the epicardial and the perirenal adipose tissue depots, which both were higher than that in the sternal ($q$<0.05) in all dietary groups. However, gene expression of *FABP3* in the sunflower-oil-supplemented group showed no difference between the epicardial and the sternal adipose tissue depots (Figure 4.13).

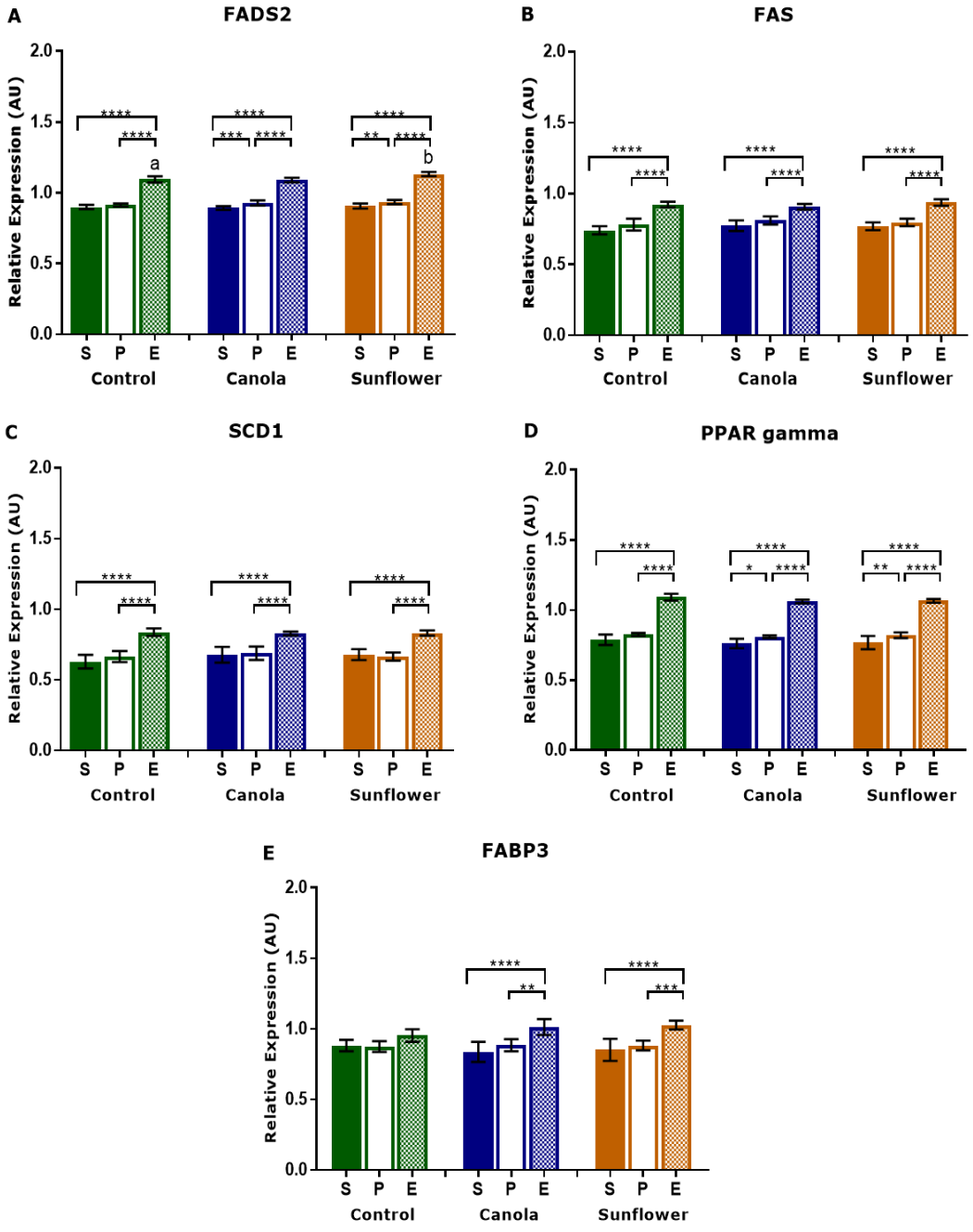



**Figure 4.12 Relative mRNA expression of genes (A) FADS2, (B) FAS, (C) SCD1, (D) PPARγ and (E) FABP3 which involved in fatty acid synthesis, storage and transfer in major adipose tissue depots at day 7 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group, and depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$q \leq 0.05$; **$q \leq 0.01$; ***$q \leq 0.001$ and ****$q \leq 0.0001$. Superscript letters denoted a significant difference between depots: a vs. b ($q \leq 0.05$).

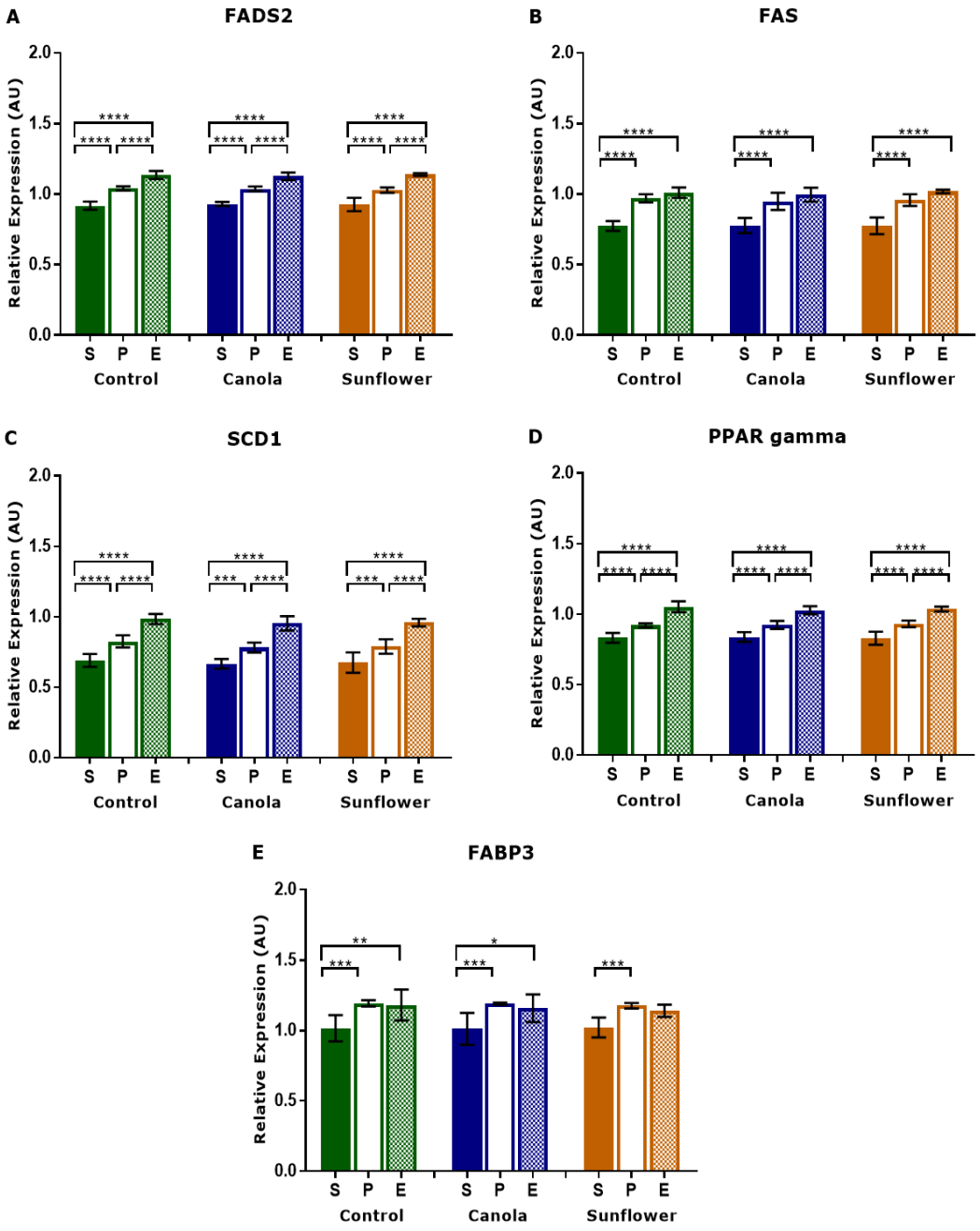



**Figure 4.13 Relative mRNA expression of genes (A) FADS2, (B) FAS, (C) SCD1, (D) PPARγ and (E) FABP3 which involved in fatty acid synthesis, storage and transfer in major adipose tissue depots at day 28 by dietary group.**

The dietary groups relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Adipose tissues are (S) sternal, (P) perirenal and (E) epicardial. All values are mean ± SEM. Relative expression in (AU) arbitrary units is normalised to the reference genes RPLP0 & KDM2B. The canola and sunflower groups were compared to the corresponding control group, and depots were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$q \leq 0.05$; **$q \leq 0.01$; ***$q \leq 0.001$ and ****$q \leq 0.0001$.

## 4.5 Discussion

Two hypotheses were tested: 1) supplementing lactating ewes with canola or sunflower oil will increase their offsprings' adipose tissue mass and the abundance of *UCP1*, and 2) the supplements will cause depot-specific variations in gene expression profile.

### 4.5.1 Effect of maternal fatty acid supplementation during lactation on offspring's gene expression

#### 4.5.1.1 Maternal fatty acid supplementation during lactation has no effect on BAT - thermogenic gene expression in suckling lambs

*UCP1* is a BAT specific uncoupling protein (Aquila et al., 1985; Cannon & Nedergaard, 2004). *PRDM 16* is a crucial transcription regulator protein in BAT and involved in *UCP1* transcription (Iida et al, 2015). It recruits EBF2-cEBPβ-PPARγ/α complex and MEDI1/Mediator complex and facilitates the folding of enhancer/promoter looping and preinitiation complex (PIC). PIC, with RNA polymerase II, initiates *UCP1* transcription (as reviewed in Seale, 2015). Therefore, mRNA expression of *UCP1* and *PRDM 16* were used as gene markers of BAT. Generally, there were no significant differences in mRNA relative expression of these genes between lambs exposed to maternal dietary supplementation during lactation and the unexposed control group at either day 7 or day 28. However, at both time points, there was little variation in mRNA expression of *UCP1* between depots in the supplemented groups and the control group. This was likely due to the relatively short duration of the dietary supplementation to produce an effect on the mRNA gene expression of the genes of interest.

Studies conducted in isolated brown-fat mitochondria suggest that fatty acids are (re)activators for *UCP1* (Shabalina et al., 2008), due to their ability to overcome the inhibition caused by nucleotides which bind to the *UCP1*, thereby inhibiting its proton transport activity and, thus, increasing proton conductance (Shabalina et al., 2004). It has been suggested that the *UCP1* uncoupling effect of fatty acid is dependent on fatty

acid level of saturation (Beck et al., 2007), structure (Matthias et al., 2000; Rial et al., 1999) and/or chain length (Bukowiecki et al., 1981; Huang, 2003; Winkler & Klingenberg, 1994), but results have been inconsistent. In humans, several studies have reported the contribution of medium-chain fatty acids to thermogenesis (Scalfi et al., 1991; St-Onge & Jones, 2003) and increased energy expenditure, compared to long-chain fatty acid supplementation (Papamandjaris et al., 1998; Saito et al., 2010; St-Onge & Jones, 2002; Tomonobu et al., 2006). Shabalina et al. (2008), in their study of BAT cells, found that caprylic (C8:0), lauric (C12:0) and palmitic (C16:0) medium-chain fatty acids demonstrated patterns of oxygen consumption stimulation. In contrast, other studies show a limited uncoupling effect of long-chain fatty acids (Schönfeld et al., 1988; Wojtczak & Schönfeld, 1993) and confirmed their role when added directly to mitochondria (Shabalina et al., 2006; Skulachev, 1999) or cells (Anderson et al., 2007; Bobyleva & Mokhova, 1994; Boudina et al., 2007). In addition, Matthias et al. (2000) and Shabalina et al. (2008) agreed on the ability of oleic acid (C18-1) to stimulate oxygen consumption in brown-fat cells *UCP1*.

As shown in Chapter 3, maternal milk's medium-chain fatty acids (i.e. 8:0, C12:0 and C16:0) were significantly decreased by the canola oil supplement at both day 7 and 28 (Section 3.4.1.2), while oleic acid increased in both supplemented groups (Section 3.4.1.3). Therefore, in view of the above data, it is unconvincing to attribute the absence of maternal fatty acid supplementation effect on the offspring's *UCP1* mRNA expression to the decrease seen in milk medium-chain fatty acids and eliminated the effect of oleic acid which has potential role in stimulating *UCP1* oxygen consumption and possibly increasing its mRNA expression. Interestingly, the *UCP1* mRNA results did not correspond to those I have found in protein abundance in perirenal adipose tissue, at least, assessed by immunoblotting in lambs fed by ewes supplemented with canola oil. This variation in *UCP1* results will be discussed further on Chapter 6 (Section 6.5.1) where *UCP1* will be investigated in more detail. It is well establish that protein abundance is a much better measure of function than mRNA (Hayles et al., 2010). Therefore, when both mRNA and protein were measured; the protein is

considered the most reliable functional measure. Therefore, these results generally fit with the original hypothesis that supplementing the maternal diet with fatty acid during lactation could increase *UCP1* expression.

Finally, it should be noted that most studies of the effects of fatty acid on *UCP1* are often performed in an isolated manner and their results, therefore, may not address any metabolic effect, (Shabalina et al., 2008). For this reason, further investigation using microarray analysis has been undertaken with the aim of finding meaningful biological insights into the effect of maternal fatty acid supplementation on offspring adipose tissue development during early life. These results are presented in Chapter 5.

#### 4.5.1.2 Elevation in lambs' circulating leptin concentrations was not accompanied by an increase in *leptin* mRNA expression

Leptin is the most characteristic marker of WAT (Pope et al., 2013). Pope et al. (2013) established the positive relationship between the maturation of adipocytes seen with age and the expression of some genes, including *leptin*. However, in this part of the study, no significant effect of maternal dietary supplementation with fatty acids on offspring gene expression was observed in the different adipose tissue depots studied. In contrast, plasma leptin was higher in female lambs compared to males in both supplemented groups at both days 7 and 28, in line with the findings of Mostyn et al. (2002). Although leptin is mainly secreted by white adipocytes (Franco et al., 2012; Siegrist-Kaiser et al., 1997), this is not the only tissue secreting it (Cammisotto & Bendayan, 2007). Therefore, the increases seen in plasma leptin in females among the groups was not unexpected. It has been reported that leptin secretion in the short term does not always result in changes in *leptin* mRNA expression (Clapham et al., 1997).

#### 4.5.1.3 The lack of change in the expression of BRITE genes is consistent with the absence of effects of fatty acid supplementation on *UCP1* expression

The mRNA expression of *HOXC9* was measured as it has been proposed to be a BRITE marker (Pope et al., 2013; Waldén et al., 2012). de Jong et al. (2015) described *HOXC9* expression as a non-informative for tissue classification in mice as it was expressed in both WAT and BRITE, but they also found that it was not expressed in BAT. In agreement with this, Yamamoto et al. (2010) reported that the expression of *HOXC9* was absent in BAT. Therefore, *HOXC9* expression can be used to make a distinction between BAT and BRITE adipose tissue and to determine whether any increase in *UCP1* might be produced by classical BAT or by the activation of BRITE adipose tissue. In addition, *SHOX2* was used as another BRITE marker, as it is highly expressed in the BRITE adipocytes, at least in mice (Walden et al., 2012).

The absence of the effect of maternal fatty acid supplementation on the mRNA expression of *HOXC9* and *SHOX2* is consistent with lack of any change in *UCP1* mRNA expression, indicating that neither canola nor sunflower oil supplements led to browning any of the three adipose tissue depots that were examined. Nevertheless, *HOXC9* mRNA expression at day 28 indicated the possibility of the presence of latent cells that are able to display brown adipocyte characteristics, particularly with the existence of an evident expression of *PRDM16* (Pope et al., 2013), in addition to *UCP1* at this time point.

#### 4.5.1.4 Expression of fatty acid metabolism-associated genes differ among major adipose tissue depots, irrespective of maternal supplementation with canola or sunflower oil

A set of genes associated with fatty acid metabolism (i.e. β Oxidation, fatty acid synthesis, and storage) were studied to gain more comprehensive insight into the effect of maternal supplementation with fatty acids during the early life of sheep. Genes examined in this part of the study included *ACOT11*, *PPARγ*, *RIP140*, *COX1*, *COX2*, *FADS2*, *FAS*, *SCD1*, and *FABP3*. Unfortunately, there are no commercially

available antibodies for use in sheep tissue, so all gene expression work was conducted using mRNA expression.

All the genes examined showed quantitative differences in gene expression among the depots which, whilst low in numerical value, showed statistically significant differences with the highest expression seen in the epicardial, and the lowest in the sternal, adipose tissue depots. Although the expression patterns observed in most genes examined were not significantly affected by the fatty acid supplementation, when each depot from supplemented groups are compared to the corresponding depot from the control group, the supplements maintained and slightly increased the differences in gene expression profiles.

#### 4.5.1.4.1 Expression of genes involved in fatty acid oxidation in the epicardial adipose tissue depot resists fatty acid oxidation

The *ACOT11* gene encodes for acyl-coenzyme A thioesterase 11, a β-oxidation pathway enzyme. It may play a role in lipid anabolism, perhaps by antagonising lipid catabolism (Ellis et al., 2015). Enzymes of the acyl-coenzyme A thioesterase family convert fatty acyl-coenzyme A to free fatty acids and coenzyme A molecules, regulate free fatty acid metabolism by controlling substrate availability during oxidation and regulate the intracellular trafficking of fatty acyl-coenzyme A (Kathayat et al., 2018). The absence of *ACOT11* expression results in an increased rate of fatty acid oxidation (Zhang et al., 2012). Thus, higher expression, as seen in the present study, favoured fatty acid synthesis rather than fatty acid oxidation. Although epicardial adipose tissue was proposed to be a depot of BAT in adult human (Ojha, 2015), it stores triglyceride to supply free fatty acid for myocardial energy production (Sacks & DaFain, 2007).

The *RIP140* gene encodes receptor-interacting protein 40. It plays a major role in blocking the expression of genes involved in energy dissipation and mitochondrial uncoupling, including *UCP1* (Debevec et al., 2007). *RIP140* knockout mice remain lean even after an energy rich diet (Leonardsson et al., 2004). These findings suggest a proactive role in fatty acid oxidation. The highest expression of *RIP140* was observed

in the epicardial, rather than the perirenal and sternal, adipose tissue depots, suggesting that fatty acid oxidation for the purpose of heat dissipation occurs less in epicardial, compared to perirenal and sternal, adipose tissue depots at least in early life.

Taken together, this suggests that the epicardial adipose tissue depot has the lowest rate of fatty acid oxidation because it is the site with the highest expression of *ACOT11* and *RIP140* genes. This is supported by further analyses of the microarray results (Chapter 5, Section 5.5) which revealed that genes associated with fatty acid metabolism were expressed more in the perirenal, than the sternal, adipose tissue depot and is consistent with the finding that genes involved in upregulating fatty acid oxidation are highly expressed in the perirenal adipose tissue of young sheep (Symonds et al., 2013).

#### 4.5.1.4.2 The potential role of *COX1* and *COX2* genes in kidney development and heart function in early life

*COX1* and *COX2* genes encode for the first and second subunits of mitochondrial cytochrome c oxidase 1 and 2 enzymes. COX1 and COX2 enzymes are reportedly involved in the oxidation of PUFA (Tao, 2015). For example, certain mutations such as Y385F lead to a failure to oxidise arachidonic acid (Marnett et al., 1999). Furthermore, blocking COX1 and COX2 enzymes causes an accumulation of some fatty acids such as dihomo-gamma-linolenic acid (DGLA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) as well as a reduction in the formation of eicosanoids (Das, 2005). As shown in Chapter 3 (Section 3.4.1.5), maternal supplementation with canola oil decreased the abundance of ω -6 long-chain PUFAs by 23% in milk, while supplementation with sunflower oil increased PUFAs and linoleic acid (LA). PUFA supplementation increases serum ω-3 and ω-6 PUFAs (Hughes et al., 2011), whilst maternal supplementation with vegetable oil enhances conjugated linoleic acid in lamb serum (Titi & Al-Fataftah, 2013). COX1 and 2 catalyse the conversion of PUFAs to prostaglandin H2 (Smith et al., 2011) which is the precursor of

five primary bioactive prostanoids that have a primary role in several physiological and pathophysiological processes in the kidney (Smith et al., 2012). Although the role of COXs in kidney development is well-established (Smith et al., 2012), their role in heart development is unknown. However, Burdan et al. (2006) reported that maternal exposure to COX inhibitors increases the rate of ventricular septal defects in rats' offspring. Additionally, prostaglandins are involved in maintaining optimal heart functioning (Beamish et al., 1985). Although *COX1* and *2* are expressed in most major organs (Smith et al., 2011), the current study found that the epicardial adipose tissue depot remained the site of highest *COX1* and *2* expression compared with other adipose tissue depots. This suggests that the epicardial adipose tissue depot is the primary site of PUFA oxidation to prostaglandins. Interestingly, although supplementation with different fatty acids could differentially alter serum PUFA profiles, my data suggest that PUFA oxidation in adipose tissue depots remain independent of fatty acid supplementation.

#### 4.5.1.4.3 Expression of genes involved in fatty acid synthesis, storage, and transfer

*FADS2* encodes for the enzyme fatty acid desaturase 2 which catalyses the desaturation of long-chain fatty acids to PUFAs (Glaser et al., 2010). Although *FADS2* is known as Δ6 desaturase, it also acts on palmitic acid (16:0) and 20-carbon fatty acids for delta-8 and delta-4 desaturations (Park et al., 2009; Guillou et al., 2003; Park et al., 2015). Xu et al. (2014) found significantly higher *FADS2* gene expression in Japanese seabass liver when the diet was supplemented with oleic acid (C18:1n-9) compared to α-linolenic acid (C18:3n-3) and a consequently higher concentration of PUFA. This indicates that PUFA supplementation could influence *FADS2* expression. Despite the increased levels of ω-6 PUFAs and linoleic acid (C18: 2n-6), and decreased levels of several other PUFAs in milk after sunflower and canola oil supplements respectively (Chapter 3, Section 3.4.1.5), altered PUFA concentrations did not affect the pattern of *FADS2* expression in adipose tissue depots between the dietary groups. Interestingly, the highest expression of *FADS2* was found in the

epicardial adipose tissue depot, which suggests this depot is the primary site of PUFA synthesis (Xu et al., 2014) and, therefore, PUFA synthesis is independent of supplemented PUFAs.

*FAS* encodes for the fatty acid synthetase enzyme which catalyses the last step in the fatty acid biosynthetic pathway (Clarke, 1993). Its main function is to catalyse the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH into long-chain saturated fatty acids (Sul et al., 1998). Several studies report a decrease in *FAS* expression in liver and adipose tissue after PUFA diet supplementation (Raclot & Oudart, 1999; Xu et al., 1999). As discussed in Chapter 3 (Section 3.4.1.5), ω-6 PUFAs and linoleic acid were raised in ewes' milk after sunflower oil supplementation whereas the concentrations of several PUFAs' decreased after canola oil supplementation. In the present study, the FAS expression pattern did not differ in adipose tissue depots between the dietary groups. However, its expression pattern did vary with age. Although *FAS* expression was highest in the epicardial adipose tissue depot at day 7 when compared with the other adipose tissue depots, the level of expression in the perirenal adipose tissue depot increased at day 28 compared to day 7, which resulted in no significant difference in the expression level between epicardial and perirenal depots. This finding suggests that the epicardial adipose tissue depot is the site of fatty acid storage in the early postnatal stage, and the perirenal adipose tissue depot becomes involved later in fatty acid storage when BAT is mostly converted into WAT.

*SCD1* encodes for the stearoyl-CoA desaturase-1 enzyme which is a Δ9 fatty acid desaturase that catalyses the synthesis of monounsaturated fatty acids (MUFAs) (Flowers & Ntambi, 2008). Saturated fatty acids (SFAs), made by *de novo* synthesis or obtained from the diet, are converted to delta-9 MUFA by stearoyl-CoA desaturase enzyme 1, which is highly expressed in the liver and adipose tissue (Yee et al., 2012) Moreover, it augments SFA-induced lipid accumulation (Matsui et al., 2012). The dietary fat components that repress hepatic *SCD1* gene expression have been established to be n–3 and n–6 PUFAs (Natambi, 1999) but not oleic acid (18:1n-9) or

stearic acid (18:0) (Hofacer et al., 2012). Although ω-6 PUFA were raised in ewes' milk after sunflower oil supplementation (Chapter 3, Section 3.4.1.5), the expression pattern of *SCD1* remained unaffected across dietary groups as the expression was significantly higher in the epicardial adipose tissue depot than the other two depots. These findings suggest that during the neonatal stage of a sheep's life, the site with the highest rate of MUFA biosynthesis is also the epicardial adipose tissue depot.

*PPARγ* encodes for the peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear transcription factor that modifies fatty acid synthesis and storage in adipose tissues (Smith, 2002). It can be activated by PUFAs such as linolenic acid (LA) (Dubuquoy et al., 2002) which was found to be increased in milk from ewes supplemented with sunflower oil (Chapter 3, Section 3.4.1.5). However, as other genes associated with fatty acid synthesis in the present study, I found that the epicardial adipose tissue depot is the site of highest *PPARγ* expression, and maternal fatty acid supplementation did not significantly affect its expression pattern in all adipose tissue depots. Laplante et al. (2003) have demonstrated that PPARγ's action upon agonist treatment was adipose depot-specific. Based on the results of the study by Laplante et al. (2003), I assumed that the activation level of PPARγ was site specific and that the epicardial adipose depot was the main site of PPARγ expression.

*FABP3* encodes fatty acid binding protein 3, also known as heart-type fatty acid binding protein 3. It is mainly a carrier protein which is involved in the intracellular transportation of long-chain fatty acids for storage (York, 2013). It is also essential for efficient BAT fatty acid oxidation and cold tolerance (Vergnes et al., 2011). *FABP3* levels are a determinant of fatty acid oxidation efficiency by BAT, and *FABP3* represents a potential target for the modulation of energy dissipation (Vergnes et al., 2011). In the present study, similar to the expression pattern observed in FAS, the epicardial adipose tissue depot was the site of highest FABP3 expression before it increased in the perirenal by day 28 of age. The perirenal adipose tissue depot could be the main site of thermogenesis in early life (see Chapter 5, Section 5.5). Similarly, in the present study, the highest expression of *FABP3* was found in the perirenal

adipose tissue depot. Since *FABP3* is also essential for efficient BAT fatty acid oxidation, this supports the agreement between the mRNA expression results and the microarray analysis (see Chapter 5).

In summary, results of the expression of genes involved in fatty acid synthesis, storage, and transfer suggest that, at least during the early neonatal stage, the epicardial adipose tissue depot is the primary site of unsaturated fatty acid synthesis. In the later postnatal stage (day 28) when BAT is mostly converted into WAT, the perirenal adipose tissue depot is involved in fatty acid storage along with the epicardial depot. Although maternal fatty acid supplementation did not affect levels of gene expression compared with controls, the expression pattern among the various adipose tissue depots was affected in some genes.

#### 4.5.1.5 Genes mRNA expression patterns differed between major BAT depots

The genes of interest examined in this part of the study, which were selected due to reasons previously discussed, clearly indicate that the different depots within BAT are not identical. All of the genes examined showed quantitative differences in gene expression among the depots, which were low in numerical value but statistically significant, with the highest expressions seen in the epicardial adipose tissue depot and the lowest in the sternal adipose tissue depot. Although the observed expression patterns of the examined genes were not affected significantly by the fatty acid supplements compared with the control group within any single depot, the supplements maintained, and slightly increased, the differences among depots in terms of gene expression profile.

Although a number of studies have shown that brown adipocytes share their developmental origins with skeletal muscle cells in their developmental origin (de Jonget et al., 2015; Seale et al., 2008; Timmons et al., 2007), the developmental relationship between adipocytes in different depots within the same adipose tissue appears to be more complex (Chau et al., 2014; Long et al., 2014; Sanchez-Gurmaches & Guertin, 2014). Based on this, it is reasonable to hypothesise that

different BAT depots will differ in their gene expression profiles, and, therefore, the brown adipocytes of each depot may differ in their characteristics. Waldén et al. (2012) detected different levels of *UCP1* expression across the interscapular (iBAT), cervical (cBAT), axillary (aBAT) and mediastinal (mBAT) brown depots in mice. Similarly, in BRITE, the *UCP1* expression was different among the epididymal (eWAT), mesenteric (mWAT) and perirenal (prWAT) adipose depots. Furthermore, different BAT depots show different levels of *HOXC9*, *Cidea* and *Prdm16* expression (De Jong et al., 2015). It has been suggested that the variation in gene expression profiles among depots within the same adipose tissue could be parallel to possible functional differences among them (Tchkonia et al., 2013). In agreement with this, Jong et al. (2015) found different gene expression profiles for WAT marker genes between the visceral and subcutaneous adipose depots. In addition, it is likely that the anatomic location and potential function of the adipose depots influenced these expression patterns.

In order to gain a deeper insight by conducting an overall analysis of the functional differences among adipose depots, rather than employing a focussed, restricted candidate gene approach, a direct comparison among major BAT depots was undertaken by performing a multi-region bioinformatics analysis (Chapter 5).

### 4.5.2 Maternal fatty acid supplementation increases offspring's adipose tissue weight

The greater adipose tissue weights observed in female lambs' adipose tissue in sunflower, and to lesser extent, canola oil supplemented groups (Section 4.4.3) partially support the hypothesis that supplementing the diets of lactating mothers with sunflower oil, and to lesser extent, canola oil would increase offspring adipose tissue mass. However, this was not achieved with male lambs. This result can be attributed to many factors, although the reason for the differential effect of sex is less clear.

Canola and sunflower oils have different mixtures of fatty acids (Chapter 3, Section 3.1.3.1), and, therefore, the milk fatty acid profile of ewes fed with each of these

supplements was different. An important difference between the two is that milk obtained from ewes fed canola oil contains numerically, not statistically compared, more ω-3 fatty acids and, in particular, α-linolenic (ALA) at day 7 and 28 respectively (results were not statistically compared in order to avoid infer subset of parameters selected based on the observed results) (Chapter 3, Tables 3.7-8). Studies of small human cohorts and in animals have shown that ω-3 fatty acids have moderate fat-lowering and anti-obesity effects (Buckley & Howe, 2010; Flachs et al., 2009). Perng et al. (2015) reported that serum ALA level is inversely related to adiposity in children, suggesting that ALA may have a potential role in protecting against weight gain; however, there is not enough convincing evidence to support this favourable effect (Lorente-Cebrián et al., 2013).

There is no direct evidence linking the association between ω-6 fatty acid intake and adipose tissue weight. Most studies proposed the association between spread of obesity in the western world over the last few decades and increase individuals' ω-6 intake, in addition to saturated fat intake (Ailhaud et al., 2006; Muhlhausler & Ailhaud, 2013). In infants, the prevalence of overweight in babies aged 6-11 months in the USA increased significantly from 1971 through 1994 (Ogden et al., 1997). This coincided with increased maternal intake of ω-6 LA, which led to an increase in its concentration in breast milk (Ailhaud et al., 2006), suggesting the potential role of maternal ω-6 linoleic acid (LA) intake in offspring adiposity. However, this association is not causation and many other factors have influence on this. For example, it has been reported that about 30% of milk LA is derived directly from dietary intake, whereas about 70% produced from maternal body fat stores (Koletzko, 2016). Thus, any factor affects maternal body fat stores will have an impact on LA concentration. However, it is not known whether the increase in total weight was due to the increase in adipose tissue weight or increase in bone mineral density (Weiler, 2000). On the other hand, mice studies revealed that maternal high fat/high ω-6 LA diet during gestation and lactation caused an increase in offspring fat mass (Massiera et al., 2003). In young mice, consumption of a high ω-6 LA diet leads to an increase in body

fat (Javadi et al., 2004). In vitro, ω-6 LA with its derivative arachidonic acid (AA) increased fat mass (Gaillard et al., 1989; Javadi et al., 2004; Azain, 2004; Muhlhausler et al., 2010). Although many in vitro studies have showed the effect of AA in increasing fat mass (Azain, 2004; Gaillard et al., 1989; Javadi et al., 2004; Muhlhausler et al., 2010), the increase in adipose tissue weight cannot be attributed to AA intake in this study as my results showed that the ω-6 AA concentration in both supplemented groups' milk was lower than that of the control group (Chapter 3, Section 3.4.1.5). In the above studies, the contrasting effects of ω-3 and ω-6 fatty acids on adipose tissue mass are shown, where the former inhibits fat deposition, while the latter promotes it. This may provide a reasonable explanation for the variation observed between the effect of canola and sunflower oils supplementation on female lamb adipose tissue weight. However, two facts challenge this hypothesis. The first is the survival of unsaturated fatty acids (USFAs) to be absorbed by the small intestine after rumen biohydrogenation, and the second is the effectiveness and efficiency of supplemented USFAs that are transferred intact from mothers to their offspring. In respect to these, my results showed no significant difference in the milk content of any ω-3 and ω-6 fatty acids, ALA and LA in particular, between the control group and the supplemented groups, except for a significant increase in ω-6 LA seen in the sunflower group (Chapter 3, Sections 3.4.1.4 and 3.4.1.5). In other words, it seems that ω-6 LA was the only survivor from biohydrogenation among the ω-3 and ω-6 fatty acids. However, although ω-6 LA increased by 15% with the sunflower oil supplementation, the milk content of its derivative AA remained unchanged compared with the control group, which means that the fatty acid supplements were not enough, or the efficiency of their conversion to their complex forms was less, than that needed to produce the desired effect. It is very likely that ALA derivatives (eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA)) produce stronger effects on adipose tissue than LA (Lorente-Cebrián et al., 2013). Therefore, further work is needed to support this hypothesis.

However, none of the above-mentioned studies adequately explain why males who received the same milk as females did not show any increase in their fat mass. It is unlikely that the milk composition produced by these males' mothers was different from that produced by the females' mothers. Many of the ewes in this study were feeding male/female twin pairs. However, it is possible that the alteration in milk components by supplementing maternal diets with fatty acids has sex-specific effects. It has been reported that genders differ in metabolism, fatty acid processing and adipogenesis (White & Tchoukalova, 2014). In humans, adult females generally have more adipose tissue than males (Blaak, 2001; White & Tchoukalova, 2014). In lambs, Wallace et al. (2014) found that 11-week-old females had a higher percentage of fat than males of the same age. The potential underlying mechanism for reduced adipogenesis in males could be deduced from the evidence that the ω-3 ALA is converted more efficiently to EPA in human females than in males (Childs et al., 2014). However, considering the competition between ω-3 ALA and ω-6 LA fatty acids on the same desaturase and elongase enzymes, it is reasonable to consider that the opposite is true. Consequently, it could be that the conversion efficiency of ω-6 LA to its derivative ω-6 AA, which is more effective in fat deposition than that of LA, is more effective in males than in females. In this context, Lohner et al. (2013) reported in their systematic review that human males have higher levels of AA in adipose tissue and lower levels in plasma lipids than females. In conclusion, the differential effect of gender on adipose tissue mass, as seen in lambs in the sunflower oil supplemented group, requires further confirmation and investigation to determine its mechanism.

### 4.5.3 Maternal supplementation with sunflower oil increases the growth rates of female offspring

Females in the sunflower group showed a significantly higher growth rate than females in the control group at day 7 of age, but showed no significant difference in this rate at day 28 of age (Section 4.4.1). It is unlikely that the initial increase can to be attributed to a 'catch up growth' function by low birth weight individuals, as there were no significant differences in the mean birth weight between dietary groups for

either sex. As mentioned in Chapter 2, Section 2.2.2, ewes were randomly allocated to one of three dietary groups immediately after giving birth with the objective being to ensure an equal distribution by number and sex among the groups, but not taking into account the average weight of their lambs at birth. Furthermore, care was taken to keep this balance between groups, as much as possible, when the time came to choose one lamb of a twin pair for sampling. Therefore, there were some variations between dietary groups in lambs' mean birth weights and sampling age (± 1–3 days), although the differences were not statistically significant. Accordingly, in order to remove confounding by these variations, growth rate was calculated in terms of the gain in body weight per day per kilogram of birth weight.

The difference in calorie intake is a possible reason for the increase observed in the female growth rate in the sunflower group. However, because the milk energy content was not measured, no effect of the supplements was seen on milk fat content. It is possible that female lambs in the sunflower group consumed more milk (milk intake was not measured) in this investigation. If so, this is likely to be a result of chance rather than 'gender bias', as previous research which looks at gender disparities in milk intake in sheep suggests, though this research is limited (Festa-Bianchet, 1988). This is supported by the absence of differences in weight gain and growth rate between genders in the control group.

#### 4.5.3.1 Increased female growth rate in the sunflower group in early life could be damaging to their health in later life

The lambs in this study were at the most rapid growth stage, which occurs in the first few weeks after birth (Réale & Boussés, 1999), as in humans (Hokken-Koelega et al., 1995; Karlberg & Albertsson-Wikland, 1995). Any acceleration in this growth accompanied by an increase in white fat depostion, could permanently affect later health (Azain, 2004) especially if it is maintained into later life (Ali, 2013). In rats, as a result of postnatal overfeeding, weight gain was accelerated, body weight was increased in adulthood and the risk of obesity (Boullu-Ciocca et al., 2005) and its

complications, such as diabetes, CVD (Plagemann et al., 1991), renal disease (Boubred et al., 2005) and cardiac fibrosis (Velkoska et al., 2008), were increased. In humans, there is increasing evidence of a positive association between excess growth rates during infancy and being overweight in later life (Druet et al., 2011; Ekelund et al., 2006; Liem et al., 2013; Monteiro & Victora, 2005). Stettler et al. (2002) reported an association between rapid weight gain during the first four months of life and an increased risk of being overweight at seven years of age, whilst Dennison et al. (2006) found the same association but between the ages of six months and four years, regardless of birth weight. In addition, it has been demonstrated that increases in both rapid weight gain in the first year of age and 'catch-up' growth between birth and two years of age are risk factors for being overweight at seven years of age (Reilly et al., 2005).

With regards to sex differences in the association between early growth rates and later obesity, studies findings were not consistent. Thoren et al. (2015) found minor differences between genders with stronger relations seen among females. In contrast, these associations were statistically significant only among males in a study done by Eriksson et al. (2001).

Based on the above, the faster growth rates seen in the sunflower group females could be a potential risk factor for obesity in later life. However, the weights of these female lambs were not significantly different than the lambs in the other groups; thus, based on the definition of rapid weight gain (Ong & Loos, 2006), their accelerated growth cannot be described as ‘catch up’ growth. Moreover, our results showed a slightly significant increase only in the first week of age, and, then, there was no significant difference between the sunflower group and the control group at the end of the first month of life, which means that this increase was only temporary and was not maintained later on. Finally, but most important of all, as the p-value was on the borderline ($q$=0.04), this may be a result due to random sampling error; thus, it is likely not to be of practical importance (Dahiru, 2008; Sellke et al., 2001).

### 4.5.4 Effect of milk fatty acid profile alteration on offspring's plasma metabolite concentration

#### 4.5.4.1 Maternal supplementation with canola or sunflower oil during lactation decreases female offspring's levels of total cholesterol

The reduction seen in the female lambs' plasma cholesterol in both supplemented groups at day 28, which was slightly greater in the canola group than in the sunflower group, compared with controls, could be explained as a result of the alteration observed in the SFAs in the ewes' milk in both supplemented groups. In humans, serum cholesterol concentrations have been shown to respond positively to dietary medium-chain saturated fatty acids (MCSFAs) content, particularly C12:0, C14:0 and C16:0 (Keys et al., 1965), and all of these FAs showed a decrease in the milk obtained from ewes fed with canola oil and, to a lesser extent, those fed with sunflower oil (Chapter 3, Section 3.4.1.2). Moreover, the effect of dietary stearic acid (C18:0), which was increased in milk of supplemented groups, has been shown to be beneficial in reducing plasma cholesterol (Bonanome & Grundy, 1988). However, the reduction in the plasma cholesterol does not indicate whether the decrease was in the plasma levels of HDL or LDL cholesterol or both, as only the total cholesterol level was measured in this part of investigation. Therefore, as a result of this limitation, it is not possible to establish whether the effect of this decrease is positive or negative. Nonetheless, it may be reasonable to assume that LDL cholesterol was decreased, at least partly, because it is generally more abundant than HDL (Lemieux et al., 2001). Furthermore, Savolainen et al. (1991), in their study of the role of sex in the effect of diet on plasma cholesterol concentration, found no differences in the HDL cholesterol levels between genders.

Assuming that the decrease seen in female lamb's plasma cholesterol was in LDL, in part at least, this is consistent with results in the literature which found the LDL cholesterol level in women to be lower than in men (Clifton & Nestel, 1992; Kovanen et al., 1979; Savolainen et al., 1991). This sex-related difference in the LDL

cholesterol level in females is likely due to the increased activity of the LDL receptor which is induced by estrogen (Kovanen et al., 1979); therefore, this is expected to disappear after menopause (LRCPEC, 1979). Furthermore, Clifton & Nestel (1992) suggested that the differences seen in baseline LDL cholesterol between genders, which accounts for about 36% of the variance seen in the rise in LDL cholesterol, could be related to differences in gender response to dietary fat. In light of this, females are less likely to be affected by myocardial infarction compared with males on the same diet (Stampfer et al., 1991).

#### 4.5.4.2 Lambs' circulating leptin level increased by dietary fatty acid supplements only in females

The plasma leptin concentrations showed an increase in female lambs fed by ewes supplemented with canola or sunflower oil. It has been demonstrated that early life nutrition affects the circulating leptin in later life (Lönnerdal & Havel, 2000; Petridou et al., 2005; Singhal et al., 2002). Serum leptin is positively correlated with the amount of adipose tissue (Friedman & Halaas, 1998), and this is relatively consistent with the increase observed in lambs' adipose tissue weight. However, although the sunflower group showed more adiposity than the canola group, plasma leptin was higher in the latter. This could be partly explained in theory in the light of the relation between leptin and appetite. Leptin is a mediator of long-term regulation of energy balance that suppresses food intake (Klok et al., 2007). In addition, it is possible that the milk's ω-6 AA in the sunflower group led to an increase in endocannabinoid activity, 2-AG and AEA in particular (Alvheim et al., 2012), which has a negative effect on leptin signalling resulting in hyperphagia stimulation (Di Marzo et al., 2001). Thus, as a result of increased in food intake, adiposity will increased. With regards to gender variation in the plasma leptin and in the context of the same theory, De Fonseca et al. (1993) suggested sex-specific effects in endocannabinoids as in CB1 cannabinoid receptors, part of the endocannabinoid system which is involved in appetite (Herkenham et al., 1991), as being more pronounced in females. Thus, it is possible that male lambs have lower levels of CB1 receptors than females, making their effect

on leptin signalling poor or non-existent, resulting in there being no effect of fatty acid supplements on leptin among males. However, the lack of sufficient evidence to support such a hypothesis in explaining gender variation in the plasma leptin warrants further research.

#### 4.5.4.3 Maternal supplementation with canola or sunflower oil during lactation has no effect on the plasma IGF-1 concentrations in offspring

Total IGF-1, which includes IGF-1 bound to IGF binding proteins, in lambs' plasma was measured at day 7 and day 28, and no effect of either canola or sunflower oil supplements was seen in either gender at both time points.

The release and synthesis of IGF-I depend on nutrition (Veldhuis et al., 1991); however, its interrelation with nutrition still needs to be fully explained (Yamamoto & Kato, 1993). Previous data from human studies about the relationship between plasma IGF-1 concentrations and BMI or fat mass vary and are even contradictory (Copeland et al., 1990; Schoen et al., 2002; Yamamoto & Kato, 1993). While many studies have found a negative association between plasma IGF-1 and body mass and/or BMI (Copeland et al., 1990; Galli et al., 2012; Pardina et al., 2010; Parekh et al., 2010; Rasmussen et al., 1995; Yamamoto & Kato, 1993), others have found no correlation. Schoen et al. (2002) did not find any relationship between IGF-1 concentration and BMI or total abdominal or subcutaneous fat, while Rasmussen et al. (1994) found IGF-1 increased inversely with abdominal fat only in females with no association with BMI in either gender. In contrast, Copeland (1990) reported a negative correlation between plasma IGF1 and BMI in males in a study that included 107 subjects from 17 to 83 years of age. In light of this, and in line with the increased fat mass seen in female lambs in the sunflower oil supplemented group, a decrease in the IGF level in this group was expected; however, it is possible that it was difficult to detect such a difference due to the small number of lambs included in this study.

## 4.6 Conclusion

In the present part of the study, female lambs fed by ewes supplemented with sunflower oil showed higher adipose tissue weights and growth rates but not total body weights. This could be due to the increase of ω -6 PUFAs in the milk received by these lambs. Although *UCP1* mRNA expression in the three adipose tissue depots was not affected by these maternal dietary supplements, further investigation for *UCP1* is addressed in the following chapters to investigate the hypothesis that supplementing the maternal diet with fatty acid during lactation could increase *UCP1* in suckling lambs. The lack of change in the expression of BRITE genes is consistent with the absence of effects of the fatty acid supplements on *UCP1* expression and suggests that any detected increase in *UCP1* abundance in further investigations, may have resulted from the role of fatty acids in retained BAT rather than recruitment of BRITE adipose tissue. Although maternal fatty acid supplementation did not affect the levels of expression of genes involved in fatty acid metabolism, the expression pattern across adipose tissue depots was affected in some genes. Finally, these results suggest that, during the early neonatal stage, the epicardial adipose tissue depot is the primary site of unsaturated fatty acid synthesis. After that, fatty acid synthesis occurs equally in both of the epicardial and perirenal depots when BAT is mostly converted into WAT.

# Chapter 5. Transcriptional analysis of adipose tissue depots during early life and postnatal development pathways modulated by dietary fatty acid supplementation

## 5.1 Introduction

Improved understanding of adipose tissue metabolism and development could be critical in preventing and/or treating obesity (Carobbio et al., 2013), especially with respect to changes in adipose tissue that occur in the immediate newborn period (Spalding et al., 2008). This period of life is characterised by raised thermogenesis in brown fat mediated by uncoupling protein (UCP) 1 (Symonds et al., 2015) in order to enable the newborn to maintain their body temperature. Thereafter, white adipose tissue (which is characterised as an energy storage depot) gradually replaces brown fat (Rockstroh et al., 2015; Symonds, 2013).

Examining developmental changes within adipocytes is necessary to configure potentially more effective treatments for obesity and its related consequences (Claussnitzer et al., 2015; Lotta et al., 2017; Spalding et al., 2008). However, despite significant progress in understanding adipocyte development, the prevalence of obesity continues to increase (Stephens, 2012). A more comprehensive understanding of the influence of anatomical location on adipose tissue's cellular composition and development could identify new pathways which may then be targeted. This can be achieved by investigating adipose tissue biology from genetic variation and genome-wide association studies (GWAS), as well as gene expression profiling and analysis, or epigenetic variation and genomic analysis (Claussnitzer et al., 2015; Lotta et al., 2017). However, despite many studies being conducted in this field, the majority have studied the function and/or development of adipose tissue depots in an isolated manner (Gesta et al., 2007) with no focus on the potential functional differences between anatomically distinct adipose tissue depots around the body and the impact of surrounding organs (Gesta et al., 2007; Lotta et al., 2017; Symonds, 2013; Symonds et al., 2015).

With the recent increases in computer power, improvement of computational strategies and accumulation of transcriptomic data, it is possible to identify primary genes based on their expression pattern across the genome (Lotta et al., 2017). In addition, genetic relationships can be expressed in a quantifiable fashion and subjected to computational algorithms for building prediction models (Diaz-Uriarte & Alvarez de Andres, 2006; Wang et al., 2006). These models are formed by identifying clustered genes with similar co-expression regulation and which are involved in similar and/or interdependent biological functions (Barabasi & Oltvai, 2004; Zhang & Horvath, 2005). Furthermore, variations in the transcriptome with maternal dietary supplementation with canola oil between similar tissues could reflect the differences in adipocyte type, function, transcriptional programme and cellular composition (Barabasi & Oltvai, 2004). As detailed in Chapter 3, maternal diet was amended by feeding the mothers a diet supplemented with canola oil in order to alter their milk composition of fatty acid (FA) (see Section 3.6, Table 3.12). Supplementing dairy ruminants with canola oil has been reported to decrease milk ratio of omega-6/omega-3 which, in turn, up-regulates UCP genes in adipose tissue (Welter et al., 2016).

In summary, progress in analysing large numbers of differentially expressed genes and the increase in the knowledge of biological systems allows interpretation of differential network analyses and taking advantage of the results with an improved understanding of adipose tissue development.

## 5.2 Hypothesis

In this Chapter, a machine learning (ML) algorithm, followed by a weighted gene co-expression networks analysis, was employed in order to obtain meaningful biological information of microarray datasets from 3 major adipose tissue depots in sheep (sternal, perirenal and epicardial) known to be populated with brown adipocytes in early postnatal life (Symonds et al., 2012). By analysing this data, it is possible to clarify the degrees of cellular plasticity in different adipose tissue depots with the aim of identifying the most adaptable adipose tissue in response to dietary intervention during lactation. Cellular plasticity indicates the cell's capacity/ability (e.g. in one depot) to take on characteristics of cells elsewhere in the body (e.g. other depots) (Vicente-Duenas et al., 2009). As shown in Chapter 3 and consistent with the literature, maternal dietary supplementation with canola oil led to a shift in the maternal milk FA composition with a decrease of omega-6, an increase of omega-9 FAs and conjugated linoleic acid (CLA) (Welter et al., 2016), which all have been found to affect the regulation of genes involved in thermogenesis (Lim et al., 2013; Peters et al., 2001; Shen et al., 2013). For this Chapter, it was hypothesised that adipose tissues from different anatomical locations in the same individual would have depot-specific gene expression signatures that reflect their capacity to differentiate and respond to dietary changes during early postnatal life. This approach provides a deeper understanding of the characteristics of different adipose tissues which could, ultimately, impact on therapeutic developments.

## 5.3 Methods

### 5.3.1 Animal Model

The experimental model used in this Chapter is summarised in Figure 5.1, while the laboratory methods are described in detail in Chapter 2, Sections 2.1 and 2.2. For this investigation, the sternal, perirenal and epicardial fat depots were collected from lambs at 28 days of age which were fed by mothers receiving their normal nutritional requirements throughout lactation (control, n=5) or their normal nutritional requirements supplemented with 3% canola oil (n=5). Tissues were quickly dissected and weighed before being sectioned and snap frozen in liquid nitrogen for storage at −80°C as described in Section 2.2.4.4.

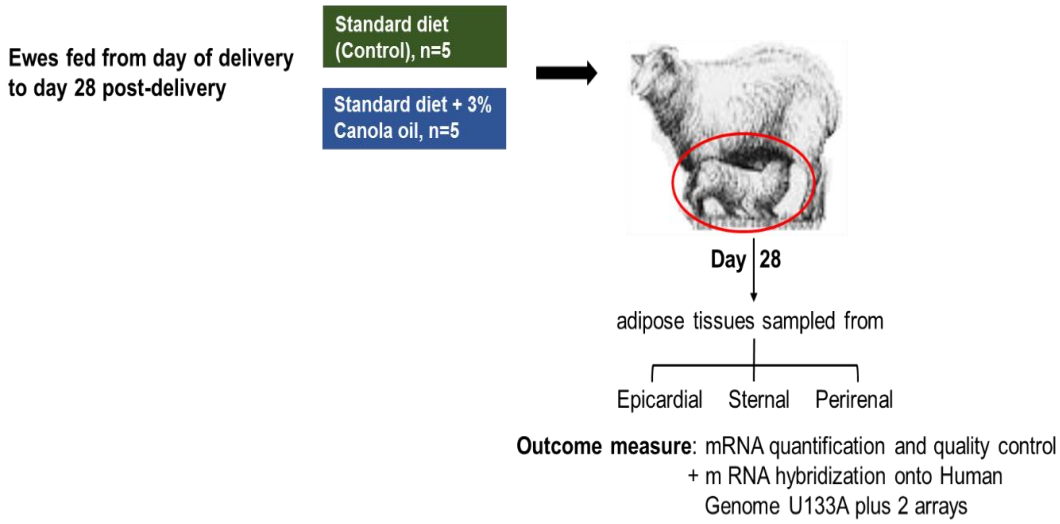



**Figure 5.1. Summary of experimental model used in Chapter 5.**

Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil. Offspring lambs were mother-fed from birth until tissue sampling day. At day 28 of age, RNA was extracted from sternal, perirenal and epicardial adipose tissues and its integrity was assayed. mRNA samples were hybridised onto Human Genome U133A, plus 2 arrays.

### 5.3.2 RNA extraction, concentration, and quality control

As described in Chapter 2 (Section 2.5.1), RNA was extracted from 1g of each adipose tissue depot sample (15 control samples and 14 samples from supplemented group, from the three adipose tissue depots extracted from the same animal). Total RNA was extracted using RNeasy kit (Qiagen, Crawley, UK) and its concentration was measured by NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, DE, USA). Optical density ratios (260/280 nm) were >2 for all samples. RNA integrity was assayed by the Agilent 2100 Bioanalyzer Eukaryote total RNA kit (Agilent Technologies, USA) and only samples which had an RIN >7 were used for microarray analysis.

### 5.3.3 Microarray-based transcriptome profiling

The mRNA samples were labelled and hybridised onto Human Genome U133A, plus 2 arrays that could be used to study ovine tissues (Graham et al., 2011). The process was performed using the GeneChip 3' IVT Express kit (Affymetrix) (Thermo Fisher Scientific Inc.; UK) according to the manufacturer's recommendations. Detection was carried out using a GeneChip Scanner 3000 7G (Affymetrix).

### 5.3.4 Expression analysis of microarrays

The quality control (QC) of entire arrays was assessed as mentioned in Chapter 2, Section 2.5.10. It is well established that assessing QC is an essential aspect of successful microarray data analysis (Wilson & Miller, 2005). Despite the expectation of arrays to show variations in their QC values, the accuracy of gene expression analysis results could be insufficient. However, it is possible to increase their accuracy by removing any outlier arrays (Wilson & Miller, 2005). Therefore, gene expression profiling was corrected in order to ensure that experimental outputs reflected definitive expression patterns and this ensured confidence in the quality of the measurement with the absence of noise and a low signal-to-noise ratio (Chen et al., 2002). This detects more genes and consequentially increases the number of genes that are statistically filtered (Wilson et al., 2004). Thus, the data were expressed in a log scale in order to account for large skewed values as well as percent changes with a

view to removing the variance effect between microarrays. The variance effect (also known as the systematic variation) is when the same gene or sets of genes from the same tissue/sample have different magnitudes of expression between microarrays.

#### 5.3.4.1 Statistical analysis

Normalisation and network analyses were performed using free and open source packages from the R project (http://cran.r-project.org/). For pre-processing data, including background correction, normalisation, and probe match verification, the open source Bioconductor community (http://www.bioconductor.org/) was used with the function "gcrma" embedded in the "affy" package. For statistical analysis of gene expression, the "limma" library was used, which allowed to perform empirical Bayesian statistical modelling among the 3 adipose tissue depots and the 2 dietary groups (control and canola oil-supplemented) (Wettenhall & Smyth, 2004). For all other statistical analyses, the false discovery rate (FDR) approach was applied with $q \leq 0.05$ considered as significant. For assessing fold changes, making heat maps, and making expression plots, the R-package "gplots" was used. I performed all data analyses with the assistance of Dr Hernan Fainberg (Department of Academic Child Health, University of Nottingham) in some specific aspect of network analyses.

### 5.3.5 Selection of informative genes

In order to identify the changes in gene function across different fat depots by one month of age, computer-assisted learning algorithms were combined with weighted gene co-expression network analysis. The optimal gene expression pattern for each adipose tissue depot in each dietary group was assessed by a reiterative process of error minimisation and supervised learning algorithms such as the random forest (RF) (Breiman, 2001). In addition, the positive sample-only learning (PSOL) algorithm developed from the RF algorithm was applied using the R package ml-DNA (Ma et al., 2014) due to its ability to achieve high specificity in the prediction of genes identification in cell biology (c. 95-98% of the informative genes) (Díaz-Uriarte & De Andres, 2006; Ma et al., 2014; Wang et al., 2006). The ML programs were able to

perform classification or prediction tasks on the top 100 genes selected through empirical Bayesian statistical modelling among individual adipose tissue of each dietary group. The accuracy of individual results was tested using the 5-fold cross-validation method (Breiman, 2001). Each selected interaction cycle was validated by assessing the values of dimension between the x and y axes at all thresholds which represent false and true positives, respectively. These dimensions, known as the area under the curve (AUC), were obtained from receiver operating characteristic analysis (ROC) and range from 0 to 1. The closer the AUC value is to 1 indicates a better prediction accuracy for the RF model.

### 5.3.6 Constructing gene co-expression networks

Datasets included data from 15 samples obtained from the control group and 14 samples obtained from canola oil-supplemented group comprising 3 different adipose tissues depots (i.e. sternal, perirenal and epicardial) from 5 animals in each dietary group (only 4 sternal adipose tissue samples from canola oil-supplemented group). These datasets were constructed individually using a standard workflow, as recommended by the weighted correlation network analysis (WGCNA) (Zhang & Horvath, 2005) and adapted by Fainberg et al. (2017). Following this, each dataset was used to constructing a signed weighted correlation network. Only 'informative genes' selected from each group by the ML-based filtering process were analysed to better describe the molecular events (Ma et al., 2014). Despite there is no clear definition of 'informative genes' term (Dziuda, 2010), it has been used in this study to refer to genes containing all of information significant for modules differentiation.

Weighted gene co-expression network describes the correlation patterns among genes across microarray samples in each dietary group. In order to assess the relationship between founded modules across dietary groups, the ‘module preservation’ function was added into the WGCNA-R package (Fainberg et al., 2017). This function calculates module preservation statistics pair-wise between reference sets (i.e. control group) and test sets (i.e. canola oil-supplemented group). For each reference-test pair, the function calculates a Z-summary value that measures how well the founded modules

in the control group set are preserved in the canola oil-supplemented group set using only the common genes between the sets. Z-summary scores >10 are interpreted as strongly preserved (that is, the architecture of these module structures is unchanged), scores between 2 and 10 are moderately preserved, and Z-summary scores < 2 are not preserved (Oldham et al., 2006).

### 5.3.7 Gene ontogeny analysis

Functional enrichment analysis was performed in order to interpret all genes listed in each module using WebGestalt (WEB-based GEne SeT AnaLysis Toolkit). The GO terms with a FDR <0.05 and enrich > 5 gene per classification are shown (Wang et al., 2013).

## 5.4 Results

### 5.4.1 Differences in the cellular landscape of brown adipose tissue in the first month of life

A transcriptomic comparison among the lamb's sternal, perirenal, and epicardial adipose tissue depots was performed in order to identify differences in gene regulation among these depots at 28 days of age. To detect the relationships among the genes expressed among the 3 adipose tissue depots, a Venn diagram was generated with overlapping circles, each of which represents a gene set expressed in two depots. The number of genes expressed uniquely in one set is located close to the sides of the circles whereas the numbers of genes expressed commonly among sets are located in the overlapping areas. Among the three depots, 1,598 genes were significantly expressed (FDR $q \leq 0.05$). The number of genes expressed were different across different replicates, with the greatest genes expressed were between sternal and perirenal fat (1,215 genes, FDR $q \leq 0.05$). Only 12 genes were regulated commonly in all depot sets (Figure 5.2).

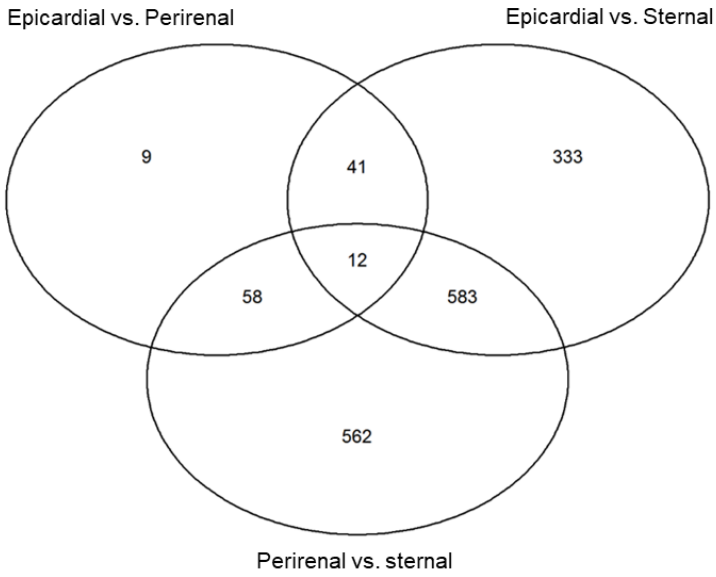



**Figure 5.2. The distribution of genes expressed among adipose tissue depots in controls.**

Venn diagram was generated with overlapping circles, each of which represents a gene set expressed in two depots. The number of genes expressed uniquely in one set is located close to the sides of the circles whereas the numbers of genes expressed commonly among sets are located in the overlapping areas. vs. stands for the versus between adipose tissue depots.

As the numbers of expressed genes varied across the replicates, it was useful to perform multiple analyses in order to correct background noise, which increases data validity. Therefore, k-means clustering and a tree analysis (dendrograms) were performed. This resulted in separation of the gene expression profiles into 3 distinct sets, each of which represents one fat depot. The analysis indicated that sternal adipose tissue showed a downregulation of gene expression compared with epicardial and perirenal adipose tissue depots. In addition, despite no significant differences among depots in UCP1 gene expression, only perirenal adipose tissue revealed a consistent upregulation of thermogenesis-related genes (Figure 5.3).

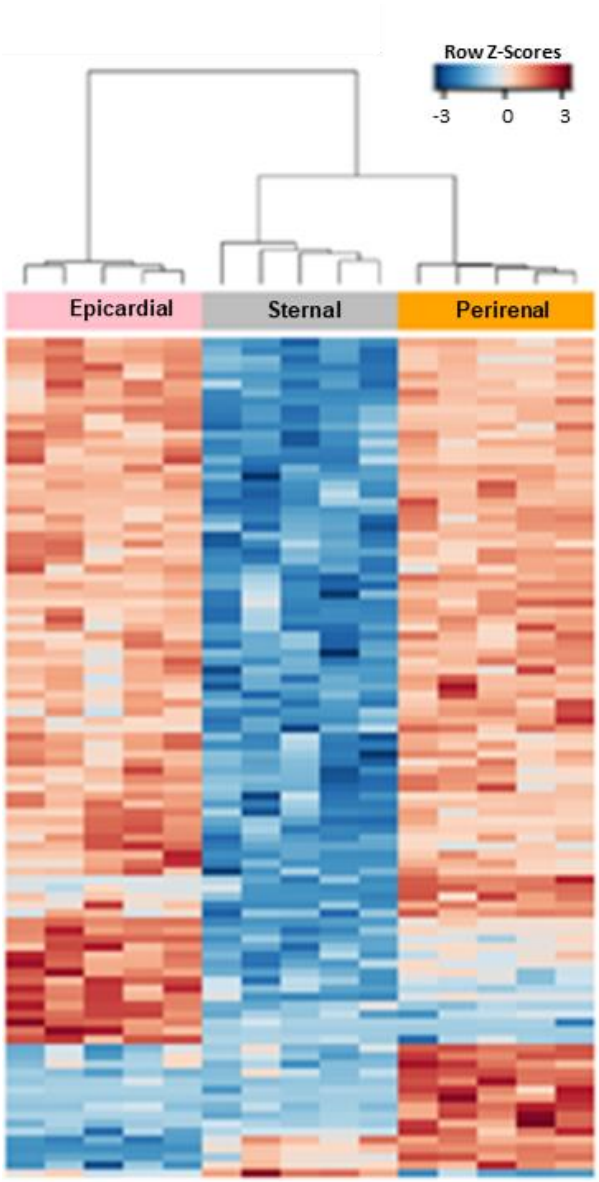



**Figure 5.3. Gene expression of brown adipose tissue depots at 28 days of age in the control group.**

The heat map shows comparisons of the top 100 genes expressed differentially among the adipose tissue depots. Gene expression was identified by microarray analysis (average linkage, Euclidean distance metric) as selected by eBayes moderated t-statistics (FDR $q < 0.05$) and was transformed into a Z-score. Blue represents a decrease, and red represents an increase in gene expression among each depot. Column colour-coding indicates adipose depots: pink - epicardial (n=5); grey - sternal (n=5); orange - perirenal (n=5).

The same statistical analyses were repeated on samples obtained from lambs fed by mothers whose diet were supplemented with canola oil to determine the changes in gene expression affected by maternal supplementation. At 28 days of age, a total of 937 genes were identified to be expressed differentially among the 3 adipose tissue depots (FDR $q \leq 0.05$), with the greatest difference between sternal and epicardial fat (670 genes, FDR $q \leq 0.05$). Only 6 genes were regulated in all depots (Figure 5.4). Similar to controls, sternal adipose tissue showed a downregulation of the transcriptional architecture in comparison with epicardial and perirenal adipose tissue (Figure 5.5). Although maternal supplementation did not influence the expression of the UCP1 gene among depots, the expression of genes involved in the regulation of energy expenditure and lipid metabolism were significantly increased.

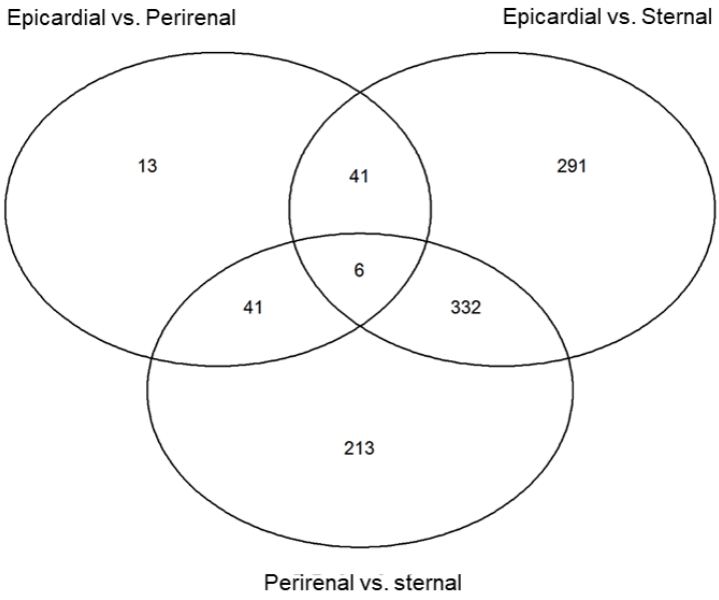



**Figure 5.4. The distribution of genes expressed among adipose tissue depots in the canola oil-supplemented group.**

Venn diagram was generated with overlapping circles, each of which represents a gene set expressed in two depots. The number of genes expressed uniquely in one set is located close to the sides of the circles whereas the numbers of genes expressed commonly among sets are located in the overlapping areas. vs. stands for the versus between adipose tissue depots.

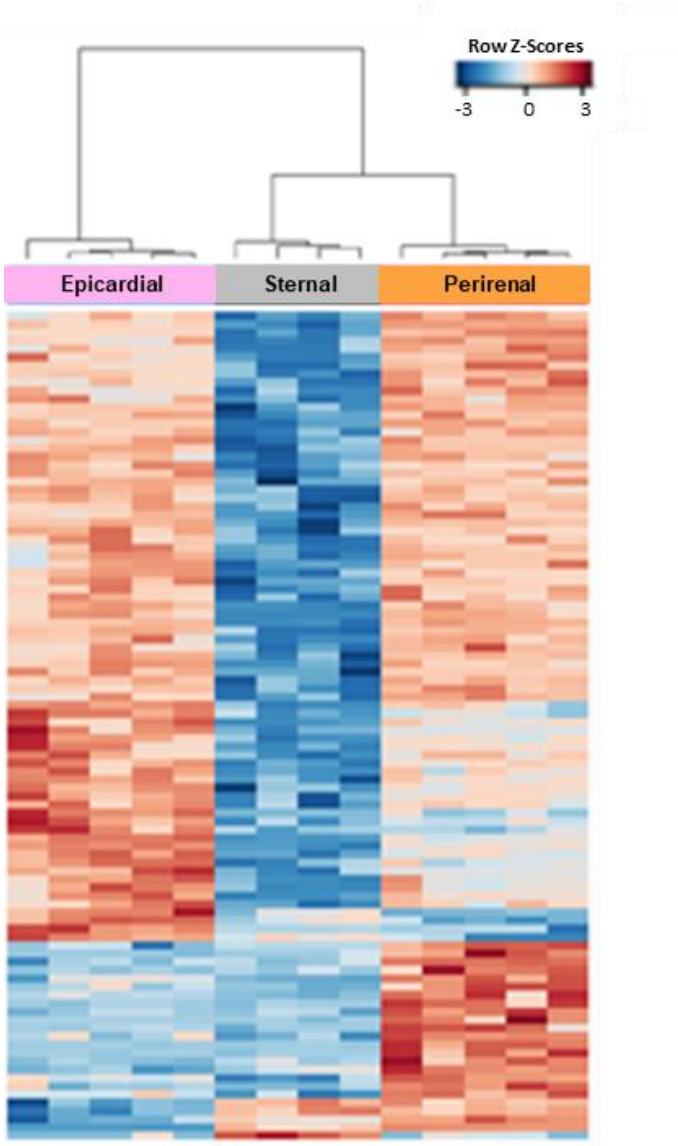



**Figure 5.5. Gene expression of brown adipose tissue depots at 28 days of age in the canola oil-supplemented group.**

The heat map shows comparisons of the top 100 genes expressed differentially among the adipose tissue depots. Gene expression was identified by microarray analysis (average linkage, Euclidean distance metric) as selected by eBayes moderated t-statistics (FDR $q < 0.05$) and was transformed into a Z-score. Blue represents a decrease, and red represents an increase in gene expression among each depot. Column colour-coding indicates adipose depots: pink - epicardial (n=5); grey - sternal (n=4); orange - perirenal (n=5).

Once again, the statistical analyses were repeated on the samples obtained from the same depot from the control and canola oil groups in order to determine any changes in gene expression pattern in each adipose tissue depot caused by FA dietary supplementation. Among the 3 depots, 10276 genes were expressed and results revealed that, unlike the epicardial and perirenal depots, the sternal adipose tissue showed upregulation of gene expression with canola oil supplemented group. However, only the perirenal adipose tissue depot showed significant differences of individual gene expression with maternal FA supplementation, with an increase in the expression of 4 genes, namely NR3C1, CYB5B, GLTSCR1L and FATR (FDR $q \leq 0.05$). The former is potentially the most important due to its direct association with inflammatory responses, lipid metabolism, cell differentiation and modulation of thermogenesis in brown adipose tissue (Figures 5.6, 5.7 and 5.8).

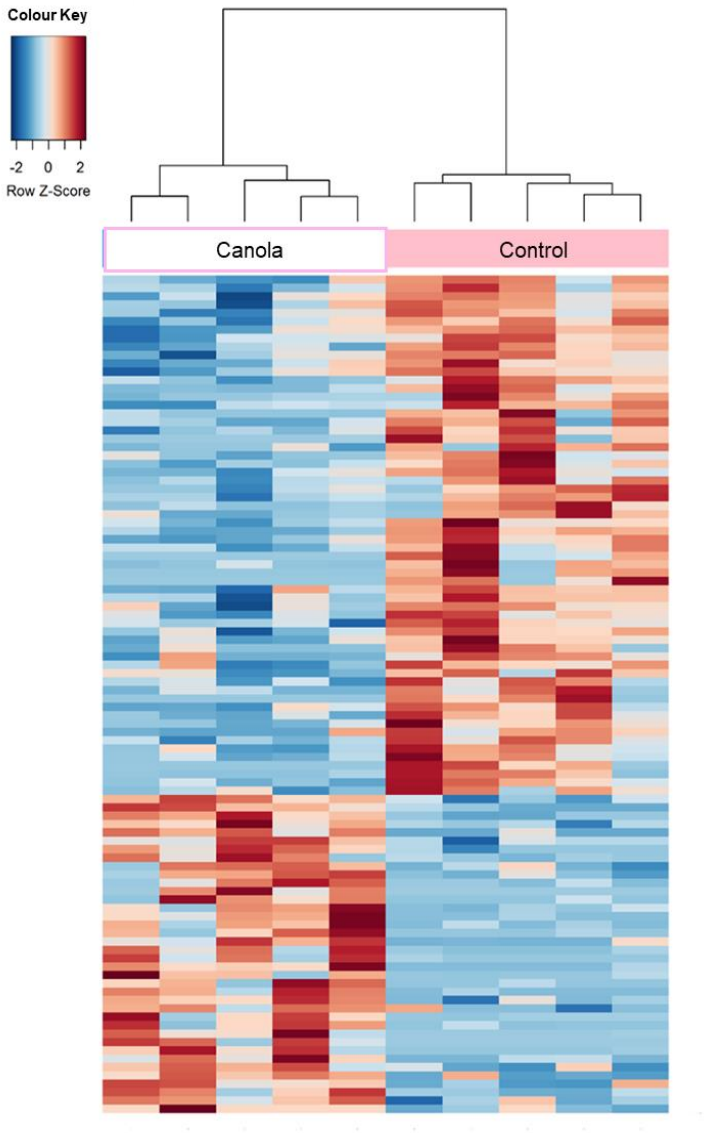



**Figure 5.6. Gene expression of epicardial adipose tissue depots at 28 days of age.**

The heat map shows comparisons of the top 100 genes expressed in the control and canola oil groups. Gene expression was identified by microarray analysis (average linkage, Euclidean distance metric) as selected by eBayes moderated t-statistics (FDR $q < 0.05$) and transformed into a Z-score. Blue represents a decrease, and red represents an increase in gene expression among each depot. Colour-coding indicates dietary group: pink - control (n=5); white - canola oil (n=5).

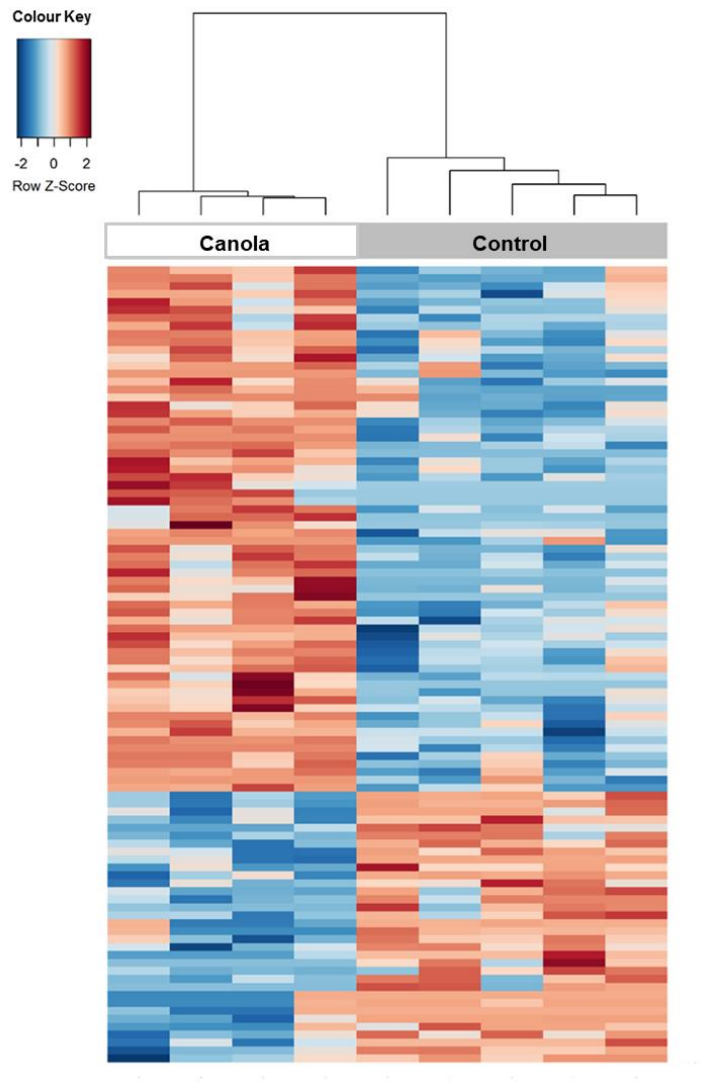



**Figure 5.7. Gene expression of sternal adipose tissue depots at 28 days of age.**

The heat map shows comparisons of the top 100 genes expressed in the control and canola oil groups. Gene expression was identified by microarray analysis (average linkage, Euclidean distance metric) as selected by eBayes moderated t-statistics (FDR $q < 0.05$) and transformed into a Z-score. Blue represents a decrease, and red represents an increase in gene expression among each depot. Colour-coding indicates dietary group: grey - control (n=5); white - canola oil (n=4).

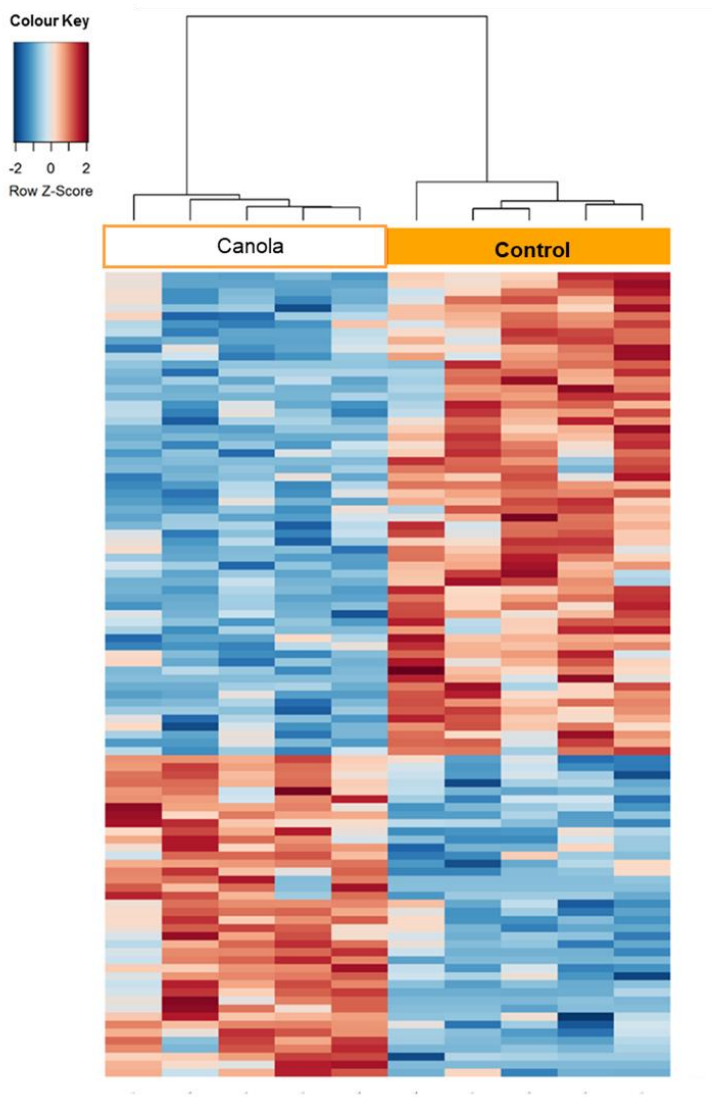



**Figure 5.8. Gene expression of perirenal adipose tissue depots at 28 days of age.**

The heat map shows comparisons of the top 100 genes expressed in the control and canola oil groups. Gene expression was identified by microarray analysis (average linkage, Euclidean distance metric) as selected by eBayes moderated t-statistics (FDR $q < 0.05$) and transformed into a Z-score. Blue represents a decrease, and red represents an increase in gene expression among each depot. Colour-coding indicates dietary group: orange - control (n=5); white - canola oil (n=5).

### 5.4.1.2 Unsupervised hierarchical clustering and principal component analysis (PCA)

A sample clustering analysis of the transcriptomic data was performed for each adipose tissue depots and showed a general level of variation in microarrays among depots. In the control group, the PCA showed that each adipose tissue depot formed distinct clusters (Figure 5.9), with a total variance in the data set of 44%, suggesting differences in gene expression among the 3 depots. In addition, perirenal adipose tissue samples were the most closely clustered, followed by sternal adipose tissue samples whereas the epicardial adipose tissue samples were the most spread out.

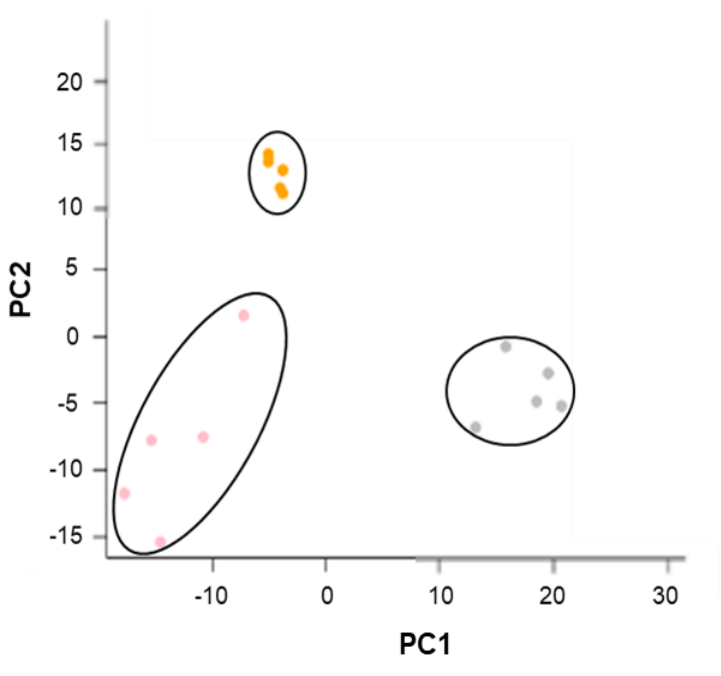



**Figure 5.9. PCA of gene expression in the control group.**

Data from the 3 adipose tissue depots from each animal at 28 days of age were analysed using 8,669 data sets that passed the variance test. Each sample is represented by a sphere and colour-coded to indicate the tissue to which it belongs: pink - epicardial (n=5); grey - sternal (n=5); orange - perirenal (n=5). PC1 axis is the first principal direction along which the samples show the largest variation. The PC2 axis is the second most important direction and it is orthogonal to the PC1 axis.

In the canola oil-supplemented group, clustering analyses showed that samples of each depot closely clustered around each other, and there was a more pronounced distinctive pattern of gene expression among the depots (Figure 5.10). Overall,

samples formed 3 separated clusters and accounted for 92% of the gene expression variance observed among the depots. This indicates that the clusters spread out from the mean and from one another, demonstrating that anatomical location influences gene expression patterns which, in turn, are modified with dietary FA supplementation.

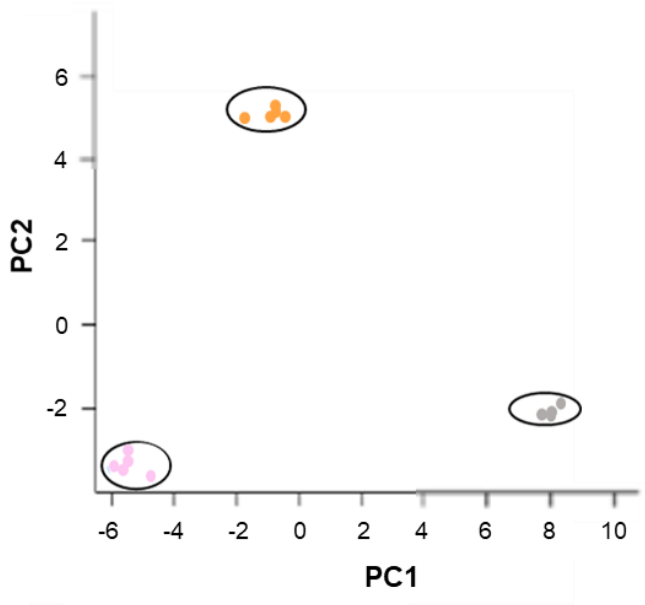



**Figure 5.10. PCA of gene expression in the canola oil-supplemented group.**

Data from the 3 adipose tissue depots from each animal at 28 days of age were analysed using 9,330 data sets that passed the variance test. Each sample is represented by a sphere and colour-coded to indicate the tissue to which it belongs: pink - epicardial (n=5); grey - sternal (n=4); orange - perirenal (n=5). PC1 axis is the first principal direction along which the samples show the largest variation. The PC2 axis is the second most important direction and it is orthogonal to the PC1 axis.

In summary, the epicardial adipose tissue depot showed the greatest transcriptomic changes which modified with dietary supplementation. This could reflect changes in biological and/or metabolic functions of perirenal adipose tissue with FA supplementation.

### 5.4.2 Changes in the transcriptional architecture among adipose tissue depots with FA supplementation

ML and gene network analyses were used in order to identify the changes in adipose tissue depot gene function induced by FA supplementation. Computer-assisted learning algorithms revealed that 2157 and 2350 genes were identified as informative genes in the control and canola oil-supplemented groups, respectively. This variation in gene numbers reflects the difference in transcriptomic response patterns in each adipose tissue depot between dietary groups.

Next, informative genes sets from each group were used individually to conduct dendrogram analysis and to generate and study a weighted gene co-expression network of the 3 tested adipose tissue depots. Figure 5.11 represents models of these genes, where each model represents specific gene sets and different biological functions. Differences in gene expression in each model were represented with colour-coding, where blue represents downregulation and red represents upregulation. This identified 8 modules of co-expressed genes in both the control (designated as C1.1-8; Figure 5.11 A) and canola oil-supplemented groups (designated as C2.1-8; Figure 5.11 B). In addition, the results showed a downregulation in gene expression pattern in the sternal compared to the epicardial and perirenal depots, with some stability in terms of gene expression during maternal FA supplementation.

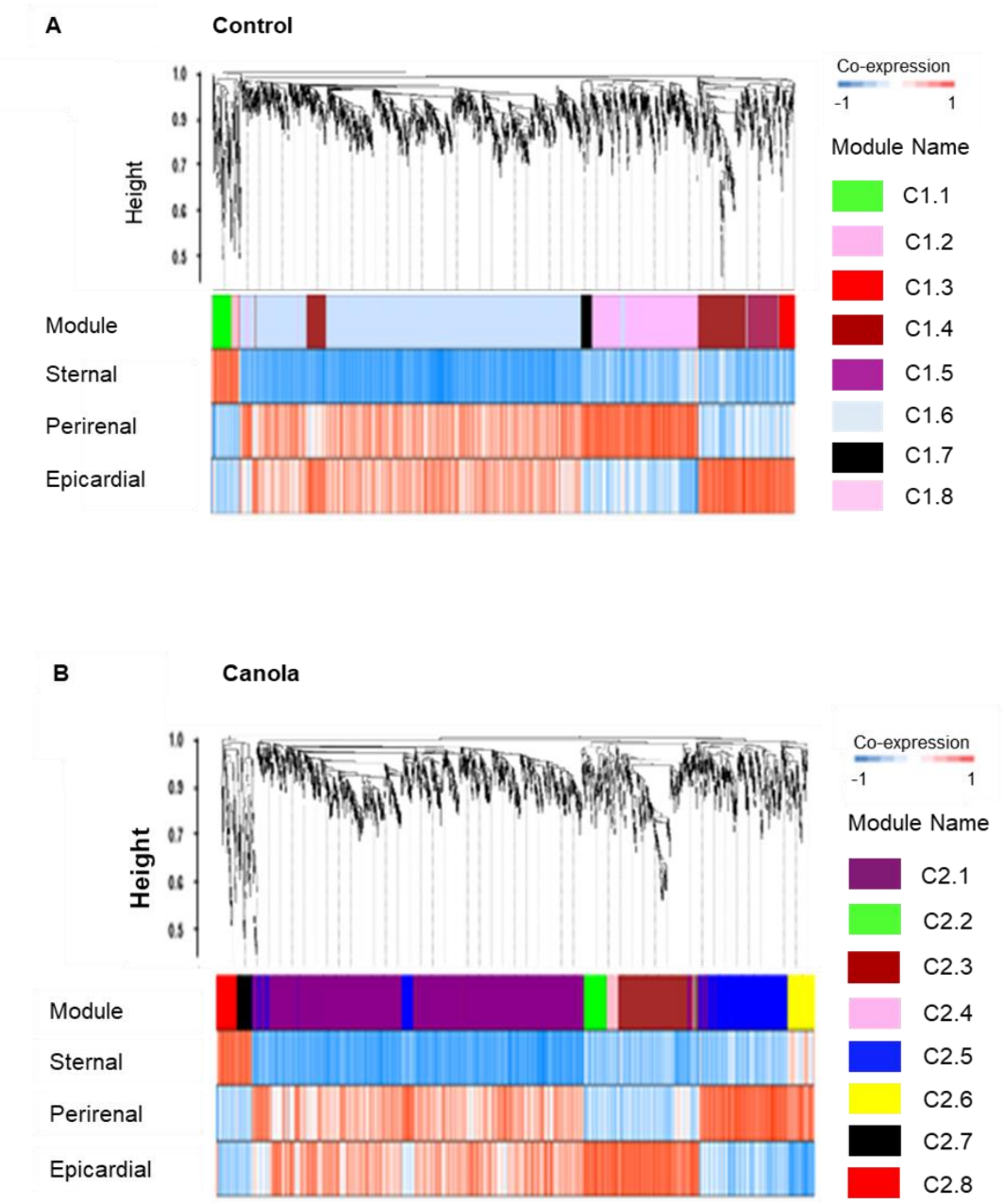



**Figure 5.11. Co-expression dendrogram analysis from the 3 brown adipose tissues included in the control (A) and canola (B) groups.**

In each dendrogram, the first row is subdivided into co-expressed modules found in each dietary group. Rows 2 to 4 show the differential expression relationships among module genes and each adipose depot. The relationship of each gene with the assigned module is colour-coded from blue (negative co-expression) to red (positive co-expression).

### 5.4.3 Functional enrichment of gene clusters

Genes network analysis utilised data-reductive techniques that generate colour-coded modules based on the topological overlap and pairwise correlations of the gene

expressions among adipose tissue depots in each dietary group. This allows for identification of the highest gene members of the module 'hub genes', which could be influenced differently by diet in different clusters within a depot and/or amongst depots. It facilitates the biological interpretation of transcriptional patterns of gene expression. Data sets from each dietary group from the 3 tested adipose tissues were constructed separately using a WGCNA method, which is commonly used for describing biological networks based on pairwise correlations between gene expression patterns and can be used to identify modules of highly correlated genes (Langfelder & Horvath, 2008). The edge weight range from 0 (indicating no interaction) to 1 (indicating a strong positive or negative interaction) (Zhao et al., 2010). Subsequent analyses of the biological functions of the hub genes in each established model in each dietary group showed some metabolic and biological differences among depots in both dietary groups, with a majority of genes within a single module corresponding to the functions of adipose tissue to which it belongs, although some were modulated by FA supplementation.

The gene network analyses demonstrated that the perirenal and the epicardial tissue depots in the control group shared some common transcriptional functionalities (Table 5.1, Figure 5.12). There were three co-expression modules with a selective enrichment in each adipose tissue depot. Some of these modules include genes involved in mitochondrial biogenesis (C 1.6 and C 1.8 in the perirenal depot, C 1.4 in the epicardial depot). Furthermore, in the perirenal adipose tissue depot, the C 1.6 and C 1.8 modules contained many genes involved in FA metabolic processes such as DECR1, GHR and PPARG which also regulates adipocyte differentiation. In addition, in the C 1.8 module, angiogenic genes, including HOXA5 and HIF1α alongside pre-adipocyte precursor genes such as HOXB6, HOXB8 and HOXB5, were co-expressed. These genes correlated with white adipose tissue expansion (Cantile et al., 2003), and their expression is consistent with the change of the perirenal adipose tissue depot function from energy expenditure to storage (Yamamoto et al., 2010). Genes enriched in epicardial adipose tissue were those in modules C 1.3, C 1.4 and C 1.5 which are associated with cardiomyocyte cell differentiation.

Conversely, all those modules were repressed in the sternal adipose tissue depot, and the only modules positively aligned were C 1.1 and C 1.2, which included a small number of genes (87 and 35 genes respectively) that were enriched with those regulating tissue angiogenesis (Figure 5.12).

**Table 5.1 Definition of the biological processes found in each co-expressed gene module in controls at 28 days of age.**

| Tissue | Module | Summary of Biological Process |
|---|---|---|
| Sternal | C 1:1 | Tissue angiogenesis |
| | C 1:2 | Tissue angiogenesis |
| Epicardial | C 1:3 | Cardiomyocyte cell differentiation |
| | C 1:4 | Cardiomyocyte cell differentiation<br>Mitochondrial biogenesis |
| | C 1:5 | Cardiomyocyte cell differentiation |
| Perirenal | C 1:6 | Mitochondrial biogenesis<br>FA metabolism process |
| | C 1:7 | NIA* |
| | C 1:8 | Mitochondrial biogenesis<br>FA metabolism process<br>Tissue angiogenesis<br>Adipose tissue expansion |

*NIA: No Information Available.

(Additional details including gene lists, average expressions and p-values can be found in appendices 3-5)

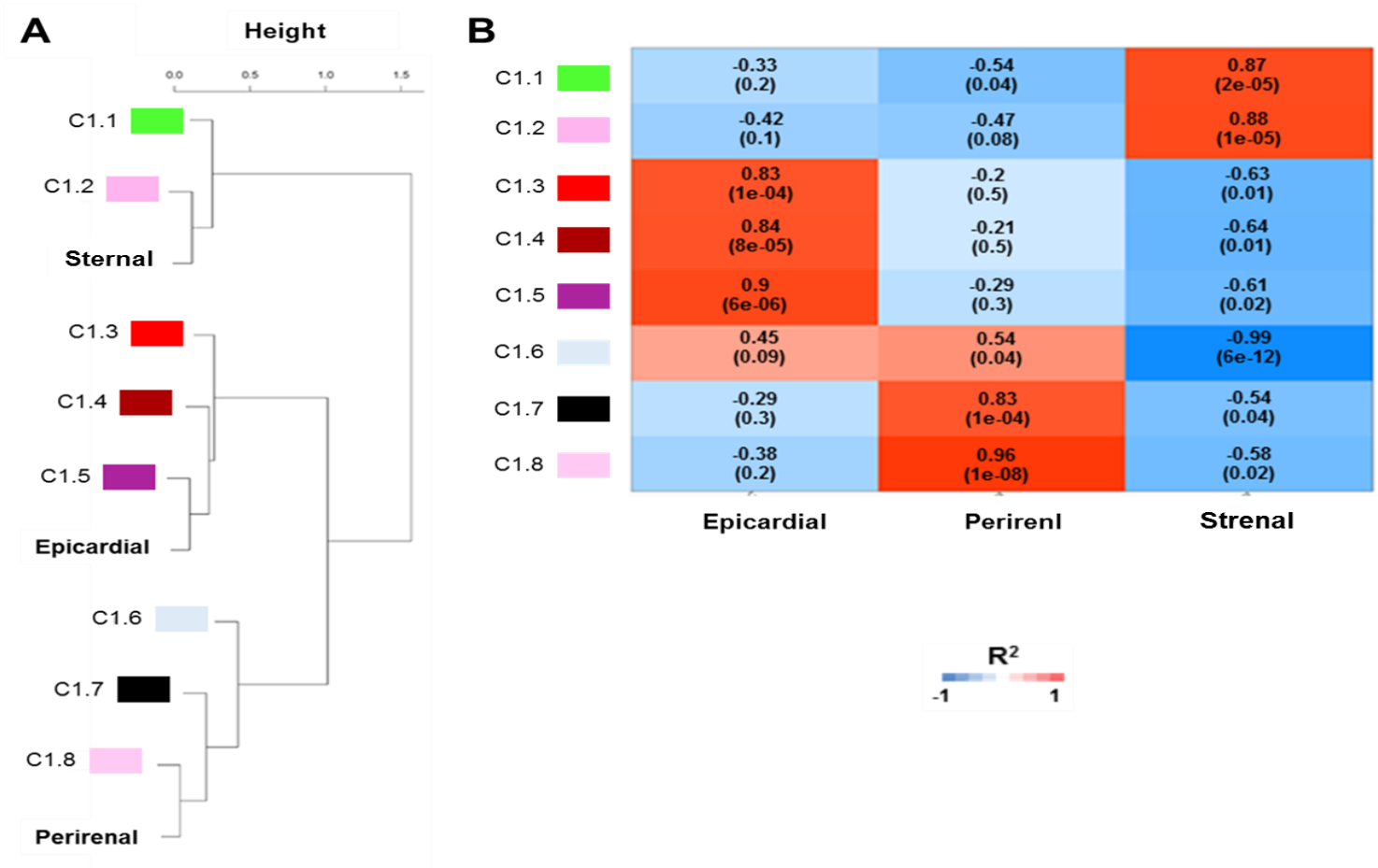



**Figure 5.12. Summary of adipose tissue depot-specific functional organisation of modules within the gene network at 28 days of age in the control group.**

Modules were related individually by their first principal component, referred to as the module eigengene (ME). Each dendrogram shows the modules of co-expressed genes and their positive alignment within the ME in each fat depot. The height (x-axis) indicates the measure of correlation expressed as Euclidean distances (B). Heat map (A) represents the correlation (and corresponding p-values) among co-expressed modules for each fat depot. The colour scheme indicates the measure of correlation, where blue = low and red = high. Regional-specific modules identified as highly correlated for each adipose depot are shown in the columns.

### 5.4.4 Changes in transcriptional control and its consequences for adipocyte function with FA supplement

To address the changes in transcriptomic regulation and its potential main phenotypical consequences for adipose tissue in the canola oil-supplemented group, another gene co-expression network was generated. As observed in the PCA, epicardial adipose tissue underwent a major shift in gene expression. Four co-expression modules were found with selective enrichment in this adipose tissue (Table 5.2, Figure 5.13). As found in the control group, these modules (C 2.1-4) contain genes that are mostly associated with cardiomyocyte cell differentiation such as RQCD1, EDF1, GDF11 and ATRAID. In addition, the C 2.1 and C 2.3 modules revealed that the genes associated with processes linked with cell division such as CDC5L, CCAR1 and FZR1 were highly expressed in the epicardial adipose tissue depot.

With FA supplementation, sternal and perirenal adipose tissue appear to share common transcriptional functionalities (Figure 5.13). Only two co-expression modules were found with selective enrichment in each of the perirenal (C 2.5 and C 2.6) and the sternal (C 2.7 and C 2.8) adipose tissues, and all contained genes functionally linked to FA metabolism. However, those genes were more active in the perirenal, than in the sternal, adipose tissues. In addition, other genes within these modules were enriched in the perirenal, but not in the sternal, adipose tissue depot were those associated with the activation of endoplasmic reticulum such as KDELR2 and SERP1.

**Table 5.2 Definition of the biological processes found in each co-expressed gene module in canola supplemented group at 28 days of age.**

| Tissue | Module | Summary of Biological Process |
|---|---|---|
| Epicardial | C 2:1 | Cardiomyocyte cell differentiation<br>Cell division |
| | C 2:2 | Cardiomyocyte cell differentiation |
| | C 2:3 | Cell division |
| | C 2:4 | Cardiomyocyte cell differentiation |
| Perirenal | C 2:5 | Activation of endoplasmic reticulum<br>FA metabolism |
| | C 2:6 | Activation of endoplasmic reticulum |
| Sternal | C 2:7 | FA metabolism |
| | C 2:8 | FA metabolism |

(Additional details including gene lists, average expressions and p-values can be found in appendices 3-5).

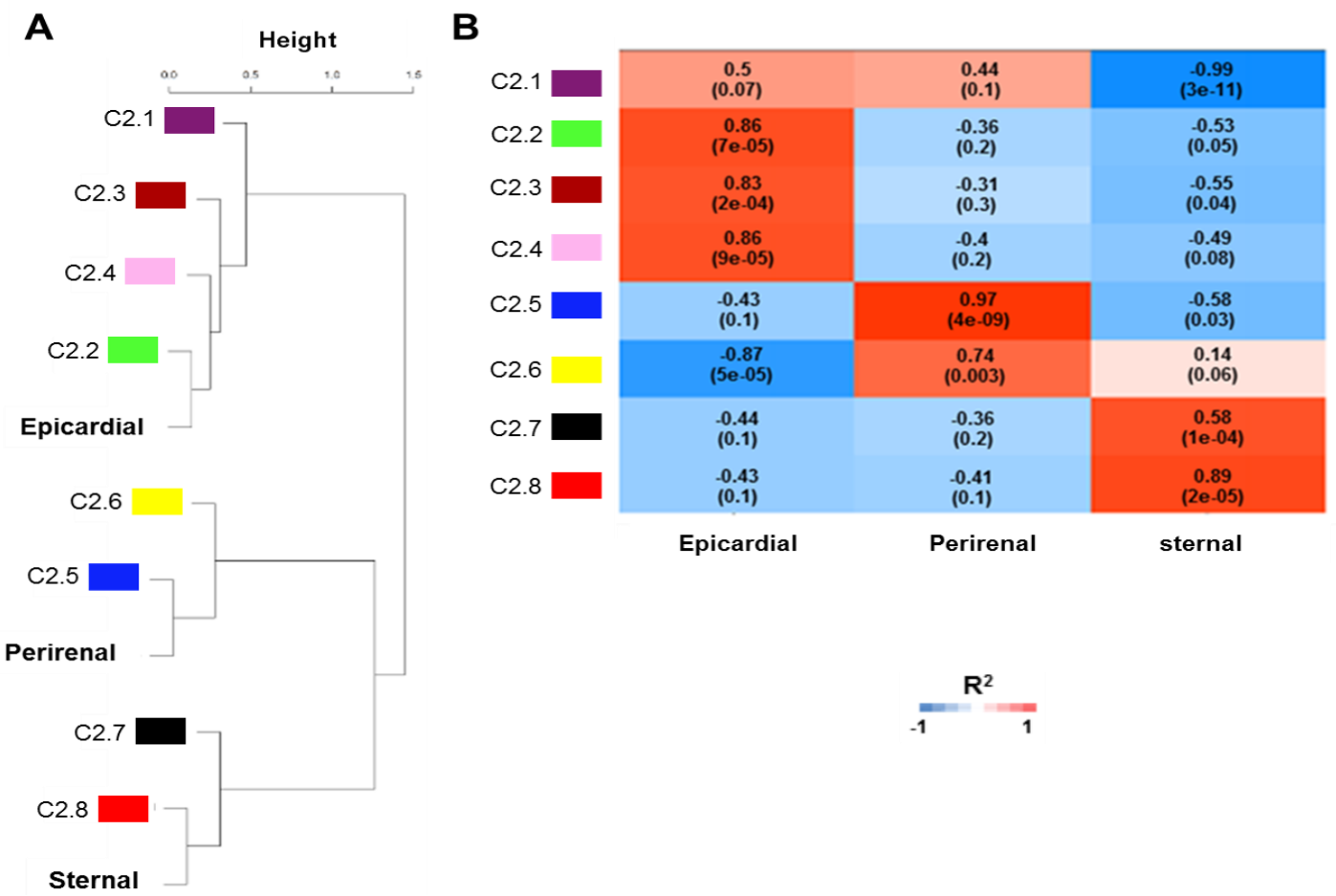


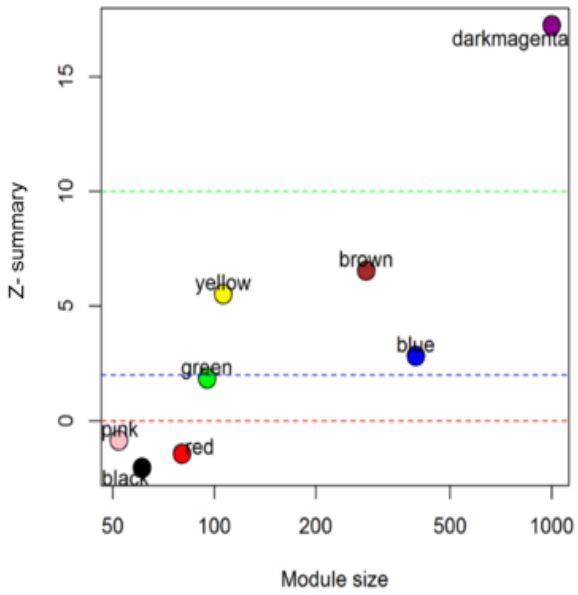

**Figure 5.13. Summary of adipose tissue depot-specific functional organisation of modules within the gene network at 28 days of age in the canola oil-supplemented group.**

Modules were related individually by their first principal component, referred to as the module eigengene (ME). Each dendrogram shows the modules of co-expressed genes and their positive alignment within the ME in each fat depot. The height (x-axis) indicates the measure of correlation expressed as Euclidean distances (B). Heat map (A) represents the correlation (and corresponding p-values) among co-expressed modules for each fat depot. The colour scheme indicates the measure of correlation, where blue = low and red = high. Regional-specific modules identified as being highly correlated for each adipose depot are shown in the columns.

### 5.4.5 Changes in gene networks represent functional alterations of different brown adipose tissue depots with FA supplement

In order to investigate the biological meaning of each module, a statistical test based on a series of random permutations between datasets, was applied to provide evidence of differentiation in the network topology (architecture of module structures).

The majority of genetic interactions persisted in both dietary groups, with 91.8% of total genes allocated to a conserved module (Figure 5.14). The gene ontogeny enrichment analysis from these modules revealed genes involved in lipid metabolism, cell differentiation and division and activation of endoplasmic reticulum.




| Module | Size | Z-summary | Adipose Tissue | Preservation | Summary of biological process |
|---|---|---|---|---|---|
| C 2.1 | 1279 | 17.2 | Epicardial | High | Cell division |
| C 2.5 | 395 | 2.8 | Perirenal | Moderated | Fatty acid metabolic processes |
| C 2.3 | 282 | 6.6 | Epicardial | | Cardiomyocyte cell differentiation |
| C 2.2 | 95 | 1.8 | Epicardial | | Cell differentiation |
| C 2.6 | 106 | 5.5 | Perirenal | | Activation of endoplasmic reticulum |
| C 2.7 | 61 | -2.1 | Sternal | No preservation | NA |
| C 2.4 | 52 | -0.9 | Epicardial | | NA |
| C 2.8 | 80 | -1.49 | Sternal | | NA |
| **Total genes** | 2350 | | | | |

**Figure 5.14. Summary of cross-adipose tissue depot module preservation with FA supplement.**

A Z-score summary of different network properties determines gene connectivity in the dietary groups at 28 days of age. Each row represents a module, and each column represents a unique feature of each module, including positive alignment with each ME and the number of genes per module. A Z-summary value of > 2 represents a moderately preserved module, and a value of > 10 provides strong evidence of module preservation. NA: not available.

## 5.5 Discussion

Adipose tissue is a metabolic organ with important functions beyond lipid storage (Coelho et al., 2013). Understanding how dietary factors, particularly during early life, affect pathways of energy utilisation or storage is an important goal in obesity prevention. However, the effects of dietary supplementation in early life on the gene expression patterns in tissues from different adipose tissue depots are largely unexplored.

As detailed in Chapter 3 (Section 3.4.1.3), supplementing a lactating mammal's diet with canola oil alters the milk FA profile, elevating the omega-9 and oleic FA content (Welter et al., 2016). Hence, I postulated that supplementing mothers' diet in this way would change gene expression profiles in the adipose tissue depots of their offspring in early life. To test this hypothesis, three major adipose tissue depots (the epicardial, the sternal and the perirenal) were examined at one month of age from lambs whose maternal diet had been supplemented with canola oil. Profound differences in gene expression profiles between the major fat depots were found, in agreement with other studies (Fainberg et al., 2017; Urrutia et al., 2016). These differences were marked, despite the similar macroscopic morphology of adipose tissues at 28 days (see Chapter 6, Sections 6.4.1.1 and 6.4.2.1), when lamb fat is primarily white (Pope et al., 2014).

Using the statistical analysis approach of Pirim et al. (2012), I found that the total number of differentially expressed genes in all depots sets was lower with maternal FA supplementation compared with controls. FA supplementation did not, however, influence UCP1 gene expression. Genes were classified, according to their similarities, using unsupervised hierarchical clustering and principal component analysis expression clustering (Qu & Xu, 2004). The results revealed that clusters varied more from the mean in the supplemented than the control group and that supplementation of the maternal diet with FA influenced gene expression.

Using a computer-assisted supervised learning algorithm, I have demonstrated that, during early life, each fat depot contained a specific transcriptome that forms a

dynamic network with unique sets of genes (Barabasi & Oltvai, 2004). Dietary alteration can cause these gene networks to undergo profound reorganisation, in which components may be added or removed (Zhang & Horvath, 2005; Ojha et al., 2016).

By examining postnatal (28 days) adipose tissues through gene network analysis, novel biological interpretations have been constructed (Barabasi & Oltvai, 2004; Zhang & Horvath, 2005) specific to each fat depot at a time when mammals were nutritionally challenged (Symonds et al., 2015). In this way, changes in gene regulation have been explored and the main regulatory relationships have been identified (Barabasi & Oltvai, 2004; Zhang & Horvath, 2005). These regulatory roles are crucial to the ability of each separate adipose tissue depot to differentiate and adapt, potentially enabling the different genes involved to modulate metabolic homeostasis (Lee et al., 2017; Macotela et al., 2012). Despite recent efforts to elucidate the cellular and transcriptome composition of different fat depots (Cypess et al., 2013; Lee et al., 2017; Lidell et al., 2013; Macotela et al., 2012; Rockstroh et al., 2015), the influence of genetic, endocrine and environmental factors on fat development remains largely unknown.

Studies of adipose tissue function during early postnatal life have mostly focused on explaining the loss of genes associated with cellular thermogenesis, especially UCP1 (Symonds et al., 2015). This study, however, aimed to discover differential adaptation between three depots at the transcriptomic level after supplementation of the ewes diet with FA in the form of canola oil, along with examining thermogenesis-related genes. In my study, it was observed that co-expression modules within different networks show a depot-specific pattern enriched with genes performing specific functions.

In the controls, k-means clustering and tree analysis (i.e. dendrograms) showed that the sternal adipose tissue exhibited downregulation of gene expression compared with the epicardial and the perirenal adipose tissue depots, in agreement with Fainberg et al. (2017). In addition, despite there being no significant differences between depots

in *UCP1* gene expression, the perirenal adipose tissue exhibited consistent upregulation of thermogenesis-related genes, while sternal and the epicardial adipose tissue depots did not. This suggests that, in comparison with other adipose tissue depots, the perirenal adipose tissue depot is metabolically more active than other adipose tissue depots. This finding was supported by that of Holloway et al. (1985). They reported that non-shivering thermogenesis was associated with increased mitochondrial GDP-binding protien and cytochrome oxidase activity in perirenal adipose tissue in adult dogs. In addition, genes involved in upregulating FA oxidation are highly expressed in perirenal adipose tissue of young sheep (Symonds, 2013).

Although McCoard et al. (2014) reported, in a study on thermogenesis in the ovine fetus, an increase in UCP1 gene expression following maternal dietary supplementation with arginine from 100 to 140 days of gestation, the present study found that supplementing the maternal diet with canola oil did not influence relative *UCP1* gene expression in the offsprings' adipose tissue depots.

A statistical analysis of control groups and canola oil-supplementation groups paired one-to-one showed that, of the three adipose tissue depots, only the perirenal depot exhibited differentially regulated genes: *NR3C1*, *CYB5B*, *GLTSCR1L* and *FATR* (FDR ≤ 0.05). *NR3C1* is a transcription factor which may contribute to obesity in humans (Dobson et al., 2001), playing a negative role in adipogenesis by regulating the expression of anti-lipogenic genes. Indeed, *NR3C1* may be a master regulator of lipid metabolism through its regulation of PPARα gene expression (Puig-Oliveras et al., 2016), which is directly associated with inflammatory responses (Oakley & Cidlowski, 2013), lipid metabolism (Dobson et al., 2001), cell differentiation (Yu et al., 2017) and the modulation of thermogenesis in BAT (Shen et al., 2017).

Little is known about the function of *CYB5B* (Neve et al., 2012). However, it is important to note that *CYB5B* is an essential component of the mitochondrial amidoxime reductase. Under adipogenic conditions, the activity of this reductase enzyme is regulated such that lipid synthesis decreases, suggesting a potential physiological role of this enzyme system in lipogenesis (Neve et al., 2012).

Trabalza-Marinucci et al. (2016) analysed the FA composition of meat in lambs challenged by maternal dietary supplementation with linseed oil for 3 weeks before parturition until 60 days postpartum. They concluded that supplementation of linseed oil could alter the lipid profile. Comparatively, it may be accepted to assume that maternal supplementation of linseed could influence FA metabolism in lambs. Accordingly, the up-regulation of genes associated with FA metabolism and of genes associated with energy expenditure in lambs after supplementation of their mothers' diet with canola oil in the present study may reflect adaptations in the offsprings' FA metabolism.

In summary, *UCP1* gene expression in the present study remained unaffected by maternal dietary supplementation with canola oil while *NR3C1* expression decreased only in the perirenal adipose tissue depot. Since *NR3C1* promotes thermogenesis, through a negative effect on *PPARα* on adipogenesis, thermogenesis in the perirenal adipose tissue depot could be inhibited by dietary supplementation. In contrast, thermogenesis in the epicardial and sternal adipose tissue depots may be unaffected, irrespective of dietary supplementation.

One caveat regarding this study, however, is that the absence of notable changes in UCP1 gene expression following dietary supplementation, particularly in the perirenal adipose tissue depot. This may be due to the decline of UCP1 abundance over the first month of life (Symonds et al., 2013). However, the absence of the effect of canola supplement on UCP1 gene expression may be attributable to the short duration of dietary supplementation or the relatively low amount of canola oil added to the diet (Mir et al.,1999; Okine et al., 2003; Welter et al., 2016).

Upon selective enrichment analysis of the control group, co-expression modules, as defined by van Dam et al. (2017), were found in each adipose tissue depot. Modules (C 1.6 and C 1.8) found in perirenal depots showed the genes involved in mitochondrial biogenesis, FA metabolic processes, such as *DECR1* and *PPARγ*, which regulate adipocyte differentiation (Gerhold et al., 2002; Puig-Oliveras et al., 2016). Furthermore, the module (C 1.8) showed a co-expression of the angiogenic genes

HOXA5 and HIF1A (Zhu et al., 2009) and pre-adipocyte precursor genes such as HOXB6, HOXB8 and HOXB5 (Seifert et al., 2015) (additional details including gene expression and p-values can be found in Appendix 3). According to the findings of Wilson-Fritch et al. (2003) mitochondrial biogenesis is associated with adipocyte differentiation. My finding complements their's by showing the modules in perirenal depot containing genes involved in those pathways.

In the epicardial adipose tissue depot, after gene network analyses, modules C 1.3–5 contain genes involved in mitochondrial biogenesis as well as genes highly associated with cardiomyocyte differentiation. It is well established that cell differentiation is associated with an increase in mitochondrial content and activity (Wanet et al., 2015). In vitro, embryonic stem cells have been found to be differentiated as cardiomyocytes in association with mitochondrial biogenesis (Wanet et al, 2015). Although mitochondrial biogenesis has been classically associated with brown adipocyte differentiation (Wilson-Fritch et al, 2003), my findings extend this to white adipose tissue growth in the case of perirenal adipose tissue depot, where there are genes involved in mitochondrial biogenesis, FA metabolism and adipocyte differentiation. Although the mitochondrial biogenesis genes were highly expressed in the epicardial depot, its contribution to the thermogenesis is not supported by my findings, as the genes associated with FA metabolism were not expressed, as far as the mitochondrial biogenesis is related with cardiomyocyte differentiation. Interestingly, in the sternal adipose tissue depot, all genes involved in the above-mentioned modules were repressed. The only two modules positively aligned were C 1.1 and C 1.2, which were enriched with a small number of genes that could lead to tissue angiogenesis such as HOXA5 (Cantile et al., 2003) and HIF1A (Park et al., 2013). Recently, we reported that the sternal adipose tissue depot was more abundant in UCP1 than in retroperitoneal fat and was maintained in adults (Henry et al., 2017). That showed a potential role of sternal adipose tissue depot in thermogenesis in adults. However, compared with perirenal and epicardial adipose tissue depots, my finding suggests that in early life sternal adipose tissue depot has a role in angiogenesis rather than thermogenesis or adipogenesis. Although increased adipose tissue vascularity is not

correlated with depot size, it was found to prevent fat expansion despite raised food intake (Brakenhielm & Cao, 2013). Xue et al. (2009) reported that increase vascularity in adipose tissue is essential for cold-induced non-shivering thermogenesis. Based on this, it is possible that increasing angiogenesis in sternal adipose tissue in early life results in increasing thermogenic capacity and energy expenditure in later life when exposed to cold challenge.

Gene co-expression network analysis of adipose tissues obtained from lambs whose maternal diet was supplemented with canola oil showed that the epicardial adipose tissue underwent a major shift in gene expression, compared with the control group. After selective enrichment, four modules (C 2.1–4) were found. These modules contain genes associated with cardiomyocyte cell differentiation, such as *RQCD1*, *EDF1*, *GDF11* and *ATRAID* (Dragoni et al., 1998; Liao et al., 2016; Loffredo et al., 2013). In addition, genes linked with cell division were found in modules C 2.1 and C 2.2, including *CDC5L*, *CCAR1* and *FZR1* (Aoi et al., 2013; Ou et al., 2014; Wang et al., 2016; Yu et al., 2016) (additional details including gene expression and p-values can be found in Appendix 4). This suggests that canola oil supplementation supports cell division and their differentiation into cardiomyocytes in epicardial adipose tissue depots.

In each of the sternal and the perirenal adipose tissue depots, two co-expressed modules were found (C 2.7 and C 2.8 in the sternal, C 2.5 and C 2.6 in the perirenal) after selective enrichment. These modules contain GHR and DECR1, genes functionally linked with FA metabolism (Lu et al., 2010; Rajakumari et al., 2013; Smith et al., 2006). In addition, the module C 2.5 contains the gene *PPARγ*, a master regulator of adipocyte differentiation (Siersbæk et al., 2010; Wang et al., 2008). The expression of those genes was higher in the perirenal than in the sternal adipose tissue depot. Finally, some genes in the modules C 2.5–8 associated with the activation of endoplasmic reticulum, including *KDELR2* (Lewis & Pelham, 1992) and *SERP1* (Yamaguchi et al., 1999), were enriched in the perirenal but not sternal depot

(additional details including gene expression and p-values can be found in appendices 3 and 5).

Unlike in the controls, each of the perirenal and sternal adipose tissue depots from lambs in the canola oil-supplemented group exhibited upregulation of genes associated withFA metabolism and adipocyte differentiation suggesting the potential role of maternal canola oil supplementation during lactation in promoting the involvement of these two depots in thermogenesis with assuming perirenal depot preference. This could be assumed because all the genes associated with FA metabolism and adipocyte differentiation were expressed in perirenal more than in the sternal adipose tissue depot. Furthermore, the potential activation of endoplasmic reticulum, in perirenal fat, could be associated with enhanced protein synthesis (Vitale et al., 1993).

In conclusion, this study showed that the perirenal adipose tissue depot is functionally more adaptable than the sternal and the epicardial adipose tissue depots due to its ability to undergo noticeable remodelling characterised by the increased expression of some thermogenesis modulation-associated genes in BAT even in the absence of significant nutritional stimulation.

## 5.6 Conclusion

Adipose tissue depots vary in their gene expression profile, differentiation ability and response to local stimulants (Fainberg et al., 2017; Lee et al., 2017). To understand the developmental origins of the molecular changes within fat depots in early life, a multi-region bioinformatics analysis was conducted on microarray data generated from three fat depots (epicardial, perirenal and sternal) sampled from lambs aged 28 days. It was hypothesised that, postnatally, each depot follows an independent path. The analysis showed that each depot was distinct in its gene expression profile and each contained a small number of unique modules of co-expressed genes associated with distinct biological processes.

Perirenal adipose tissue exhibited a greater tendency to differentiate in response to maternal diet, while the sternal and the epicardial adipose tissues showed lower capacity for plasticity, gene expression being actively repressed in the sternal depot. Following maternal dietary supplementation with canola oil during early postnatal life, the perirenal adipose tissue depot was the most responsive.

The differenced in depot specific functions of major adipose tissue depots in sheep (sternal, perirenal and epicardial) and factors influence these depots modulation is not well established. However, I have shown for the first time the potential effect of anatomical location on adipose tissues development. In conclusion, the application of machine learning algorithms followed by weighted gene co-expression network analysis provided a valuable tool for understanding the development of adipose tissues in early life and increasing our knowledge of adipose tissues in different anatomical sites.

# Chapter 6. Nutritional manipulation of mitochondrial protein abundance in adipose tissue

## 6.1 Introduction

### 6.1.1 Overview

In Chapter 3, the effects of supplementing a maternal diet with canola or sunflower oil on milk fatty acid (FA) profiles were tested, and the results were summarised in Table 3.12. In Chapter 5, it was demonstrated that individual adipose depots have distinct pathways of postnatal development that may be affected by many factors, including dietary supplementation.

This chapter investigates whether these developmental changes coincide with changes in the mitochondrial protein abundance in lamb adipose tissue during early life, which may affect the level of energy metabolism within the adipocyte. Specifically, it explores the effect of age and diet on these potential changes. As concluded in Chapter 5, adipose tissue depots show a different response to dietary stimulus; further investigations on protein level may enable one to distinguish between BAT depots, which could then help in targeting the most appropriate adipose tissue depot for nutritional or therapeutic interventions.

### 6.1.2 Mitochondrial proteins

Uncoupling protein (UCP) 1 is a BAT-specific mitochondrial inner membrane transport protein that, when activated by long-chain fatty acids (LCFAs), mediates non-shivering thermogenesis in mammals' BAT (Aquila et al., 1985; Cannon & Nedergaard, 2004; Enerbäck et al., 1997; Heaton et al., 1978). Upon activation, UCP1 increases the conductance of the inner membrane of mitochondrial and dissipates the mitochondrial proton gradient which accumulates through the respiratory chain, and results in the transportation of protons back to the matrix, increasing heat generation by BAT mitochondria instead of producing ATP (Fedorenko et al., 2012; Jastroch et al., 2010;

Nicholls & Locke, 1984; Sluse et al., 2006). As detailed in Chapter 1, UCP1 plays a key role in the maintenance of body temperature and control of energy balance. Another mitochondrial protein is the voltage-dependent anion channel (VDAC), which is located in the outer membrane of the mitochondria. It acts as a 'gatekeeper' that controls metabolite entry and exit to/from the mitochondria. Furthermore, it regulates mitochondrial metabolic and energetic functions and supplies ADP for energy production (Shoshan-Barmatz et al., 2010). In addition, cytochrome c is a protein located in the mitochondrial inter-membrane space and transports electrons between complex III and complex IV in the respiratory chain (Shoshan-Barmatz et al., 2010) (Figure 6.1). It is well established that an abundance of UCP1, VDAC and cytochrome c can be found after birth, sharply peaking before declining gradually with age (Mostyn et al., 2003; Symonds et al., 2013 & 2015). The ability of maternal FA supplementation to alter mitochondrial protein abundance in adipose tissue and the sustainability of this effect  need further research.

Adipose tissue depots transform from brown to white; in parallel, UCP1 loss is a metabolic adaption as adipose tissue switches from spending to storing energy, a process determined by maternal nutritional and endocrine conditions (Symonds et al., 2013 & 2015).  Budge et al. (2000) reported an increase in the abundance of UCP1 in fetuses with increased maternal food intake in late gestation. Moreover, restricted maternal food intake during the last month of gestation results in increased abundance of VDAC and cytochrome c in the perirenal adipose tissue of twin offspring (Budge et al., 2003). Therefore, changes are expected in the abundance of these proteins in suckling lamb adipose tissue following maternal FA supplementation. The effects of maternal FAs supplementation on offspring BAT mitochondrial protein abundance, to my knowledge, has not been investigated in mammals.

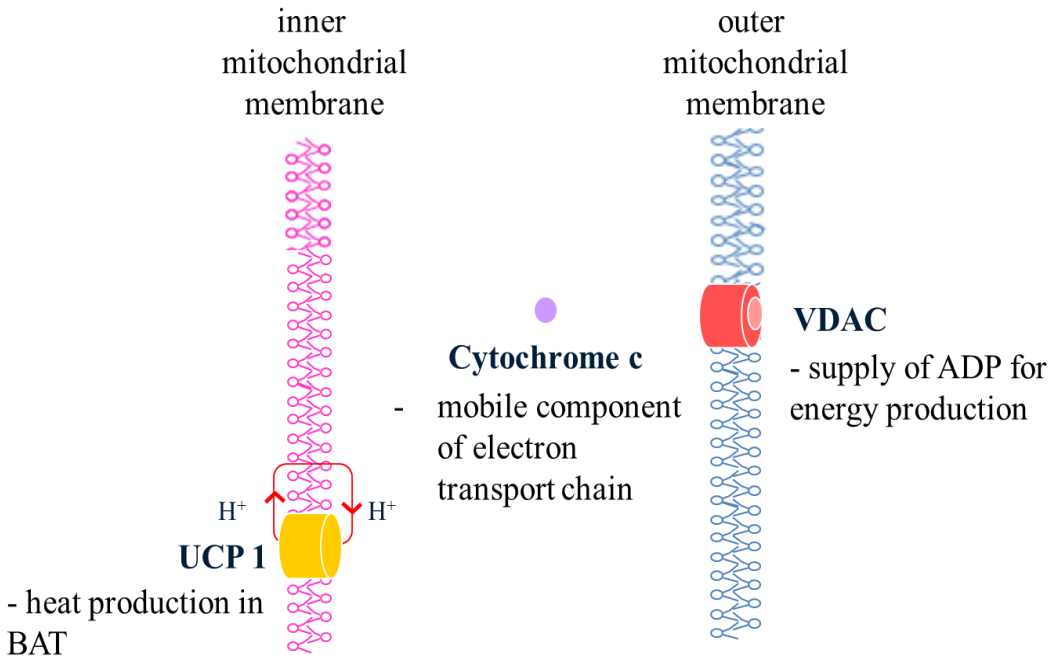



**Figure 6.1 Mitochondrial proteins and heat production.**

Mitochondrial proteins that control the energy metabolism within the adipocyte include UCP1, voltage-dependent anion channel (VDAC) and cytochrome c. Adapted from Symonds et al. (2015).

### 6.1.3 Elevation of UCP1 expression by fatty acids

Large mammals adapt to early life challenges, either environmental, nutritional or physiological (Symonds et al., 2015), and different anatomical adipose tissue depots respond differently to these challenges (Ojha et al., 2016; Rockstroh et al., 2015).

Dietary FA manipulation could increase UCP1, as shown through in vitro and in vivo studies in humans (Fleckenstein-Elsen et al., 2016; Soares et al., 2004) and rats (Priego et al., 2013; Rodríguez et al., 2002). However, the specific mechanisms by which this happens are unknown.

It should be noted that although the results of the current investigation can be attributed to a maternal diet supplemented with canola and/or sunflower oil, it cannot be confirmed as to which specific FAs are responsible for the effect on the offspring due to the many changes that occur in the milk FA profile (Chapter 3). Even so, this

investigation will help to define whether dietary FA composition can affect BAT function in early life.

## 6.2 Hypotheses

As detailed in Chapter 1, it is well established that UCP1 abundance reaches a peak immediately after birth and then declines until disappearing by 30 days of age (Clarke et al. 1997 a, b; Lomax et al. 2007; Symonds et al., 2013 & 2015). The present chapter aims to investigate whether the loss of UCP1 abundance over the first month of life runs in parallel with the loss of VDAC and cytochrome c in adipose tissue and whether the different adipose tissue depots are different in this aspect.

As discussed in Chapter 3 and summarised in Table 3.12, FA supplementation altered the FA composition of maternal milk. In agreement with research suggesting that FAs are activators for UCP1 regulation (Beck et al., 2007; Huang, 2003; Matthias et al., 2000; Shabalina et al., 2004 & 2006; Winkler & Klingenberg, 1994), I hypothesised that maternal FA supplementation during lactation would result in the up-regulation of UCP1 within these tissues compared with the controls.

As concluded in Chapter 5, and consistent with the literature, each individual adipose tissue depot has a distinct expression profile (Macotela et al., 2012; Rockstroh et al., 2015; Spalding et al., 2008). Therefore, I hypothesised that the expression of the examined mitochondrial proteins, UCP1 in particular, may differ between adipose tissue depots, regardless of the dietary intake.

## 6.3 Methods

A detailed description of the study design can be found in Chapter 2, Section 2.2. An overview of the experimental model used in this chapter is summarised in Figure 6.2. Details of the laboratory procedures (e.g., tissue histology, immunohistochemistry and Western blotting) are described in Chapter 2, Sections 2.3 and 2.4.

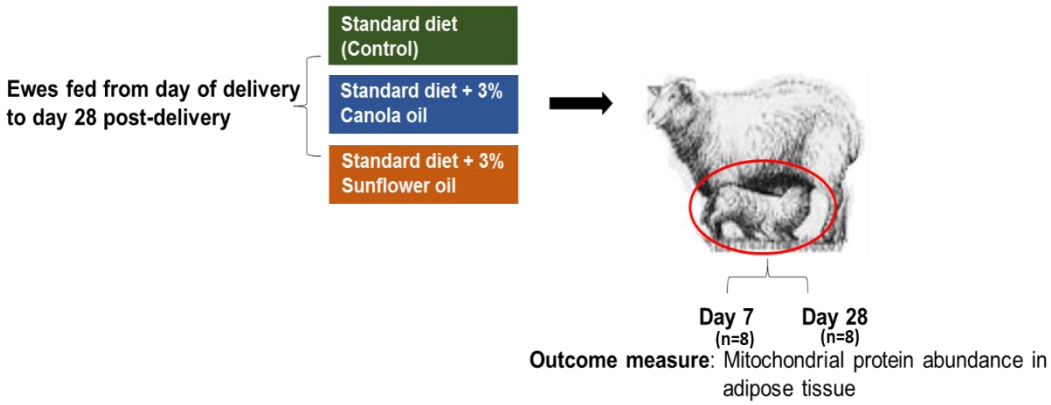



**Figure 6.2 Summary of the experimental model used in Chapter 6.**
Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil (*n*=8 per sampling age). Offspring lambs were mother-fed from birth until tissue sampling day. Day numbers refer to days of age. Within a day's sampling, the mean of each outcome in each supplemented group was compared to the corresponding control group; and within each dietary group, adipose tissue depots were compared by one-way ANOVA unless otherwise stated.

### 6.3.1 Tissue Protein Analysis (Western Blotting)

In this study, the changes in UCP1 abundance, VDAC and cytochrome c were determined in the sternal and perirenal adipose tissue in lambs aged 7 and 28 days (*n*=8 per sampling age). Lambs were fed by ewes and received their normal nutritional requirements throughout lactation (control) (*n*=8; 4F, 4M) or a revised supplemented diet with 3% of either canola or sunflower oil (n=8; 5F, 3M). In the control group, samples from the lambs at the age of 1 day (*n*=5; 4F, 1M) were also taken for comparison.

It has been established that the response of adipocytes to a local environmental stimulation differs between adipose tissue depots (Sidossis et al., 2015); which could reflect changes in the mitochondrial function of those depots over time (Henry et al., 2017; Symonds et al., 2016). The abundance of UCP1 was determined in 4.2 μg/μl mitochondrial protein using a raised in rabbit against sheep antibody prepared 'in house' (Schermer et al., 1996) at a dilution of 1 in a 10000. The abundance of cytochrome c was determined using a dilution of 1 in a 1000 of an antibody ('Santa Cruz', Santa Cruz, CA, USA). VDAC abundance was determined using an ovine-specific antibody prepared 'in house' at a dilution of 1 in 2000 (Mostyn et al., 2003). A single band at the exact molecular weight of each antibody, using molecular-weight markers on all gels, was detected in the sternal and perirenal adipose tissue (Figure 6.3). Negative (sheep liver) and positive controls (1-day-old lamb perirenal tissue) were run on the same gel to confirm that the bands detected in the adipose tissue samples were correct. For each antibody, a band with an identical molecular weight as the examined antibody (UCP1 33 kDa, VDAC 31 kDa, cytochrome c 12 kDa) was detected in all samples, except the one that had been prepared from the sheep liver. This was followed by confirmation of the specificity of the detection using secondary anti-mouse and rabbit antibodies. Each membrane was imaged, and the densitometry of the bands was analysed. The results were compared to a reference mitochondrial sample that was loaded on each gel (see Appendix 2). All results are expressed in densitometric units.

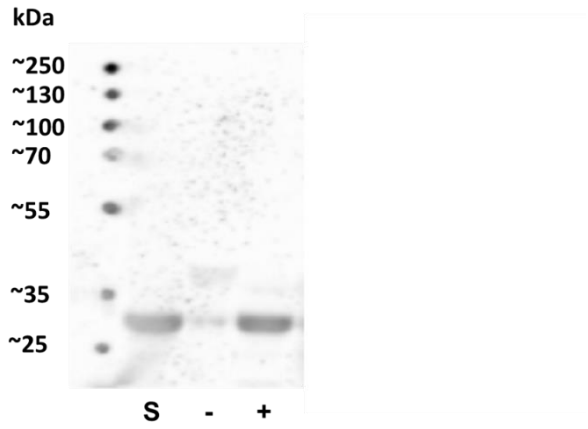



**Figure 6.3 Representative CCD-camera image of UCP1 in mitochondria.**
Samples prepared from (S) sternal adipose tissue sampled from 7-day-old lamb (-) sheep liver and (+) perirenal adipose tissue sampled from 1-day-old lamb. No protein detected for the liver sample. S and + bands are at the correct molecular weight and are, therefore, UCP1.

### 6.3.2 Histology and Immunohistochemistry

The unique marker of BAT, UCP1 was further investigated using histology and immunohistochemistry techniques to detect and quantify the abundance of the protein, as detailed in Chapter 2, Section 2.3. This was conducted on sternal, perirenal and epicardial adipose tissues, which are known to be brown in early postnatal life (Symonds et al., 2012).

In summary, formalin-fixed tissue samples were rehydrated then embedded in paraffin wax using a Shandon Excelsior™ advanced tissue processor (Thermo Shandon Ltd, Runcorn, Cheshire, UK). Five-micrometre tissue sections were cut from the samples using a sledge microtome (AS200; Anglia Scientific, Cambridge, UK) and mounted on to Superfrost™ Plus glass microscope slides (Menzel-Gläser; Gerhard Menzel, Braunschweig, Germany). Samples were stained using haematoxylin and eosin (H&E) and UCP1 antibody. At least 10 slides per animal, alongside the negative and positive controls, were labelled and randomly loaded into a Leica BondMax™ IHC slide processor (Leica Microsystems) and then run on an automated software programme (Vision Biosystems Bond version 3.4A, Leica Biosystems, Newcastle Upon

Tyne, UK) using a Bond Polymer Refine Detection reagent (Leica Biosystems, Newcastle, UK) and a 1:750 dilution of home-set primary antibody against UCP1 (Schermer et al., 1996) for the antibody-positive samples. In addition, samples were stained for VDAC using a 1:500 dilution of an ovine-specific antibody prepared 'in house' (Schermer et al., 1996).

Slides were imaged using a charge-coupled device and high-speed colour camera (Micropublisher 3.3RTV; QImaging, Surrey, BC, Canada) and then analysed with Volocity quantification software (v6.1.1, Improvision Ltd, Coventry, UK). For UCP1 quantification, the slides were scanned using NDP NanoZoomer digital pathology (C9600-02, Hamamatsu Photonics Limited, Hertfordshire, UK) and analysed using NDP.view2 Viewing software U12388-01 version 2.6.13 (Hamamatsu Photonics UK Limited, Hertfordshire, UK), as previously described in Chapter 2, Section 2.3.4.3.

The size of white adipocytes was calculated at days 7 and 28 of age for sternal, perirenal and epicardial adipose tissue samples, as detailed in Chapter 2, Section 2.3.3.2. Each cell within each selected image from the three depots was counted, as detailed in Section 2.3.4.3.1.

### 6.3.3 Statistical analysis

The results are presented as mean ± SEM. For statistical analysis, one-way ANOVA and Bonferroni post-hoc test were performed to compare between the age groups in the controls and between supplemented groups with the corresponding control group. In addition, these analyses were performed for the comparison between the two adipose tissue depots within the same age. For the study, p values $\leq 0.05$ were considered significant.

The analysis of the histology and IHC images were performed using the free open access software ImageJ (http://rsbweb.nih.gov/ij/). For statistical analysis, the mean of each intervention group was compared to the corresponding control group by one-way ANOVA and Bonferroni post-hoc test, and a $p \leq 0.05$ was considered significant.

## 6.4 Results

### 6.4.1 Effect of age on mitochondrial protein abundance in adipose tissue

#### 6.4.1.1 Histological changes in adipose tissue development with age

Tissue sections from sternal, perirenal and epicardial adipose tissue samples obtained from lambs at days 1, 7 and 28 of age were stained with H&E and for UCP1 to compare tissue morphology and UCP1 abundance. In addition, sternal and perirenal sections were stained for VDAC. Representative samples from tissues at each time point are shown in Figures 6.4 and 6.5.

There was a marked transition in the histological appearance of the tissue between each sampling age, such that the three depots showed clear transference from brown to white fat. On day 1, cells with the appearance of brown adipocytes were clearly visible and showed maximal UCP1 abundance. At day 7, no differences were seen in the histological appearance of the tissue between depots. As expected at this time point, all adipose tissue samples contained both brown, multilocular and white, uni-locular cells (Pope et al., 2014). Then, there was a decline in brown adipocytes at day 7, which continued to decline until day 28 when nearly all the white cells were distinct. VDAC was highly abundant on the first day of birth then it gradually decreased with postnatal age, closely correlated with the loss of UCP1.

In summary, a transition with increasing age was observed: UCP1 expressing brown adipocytes disappeared and were replaced by lipid-filled white cells that increased in size with age.

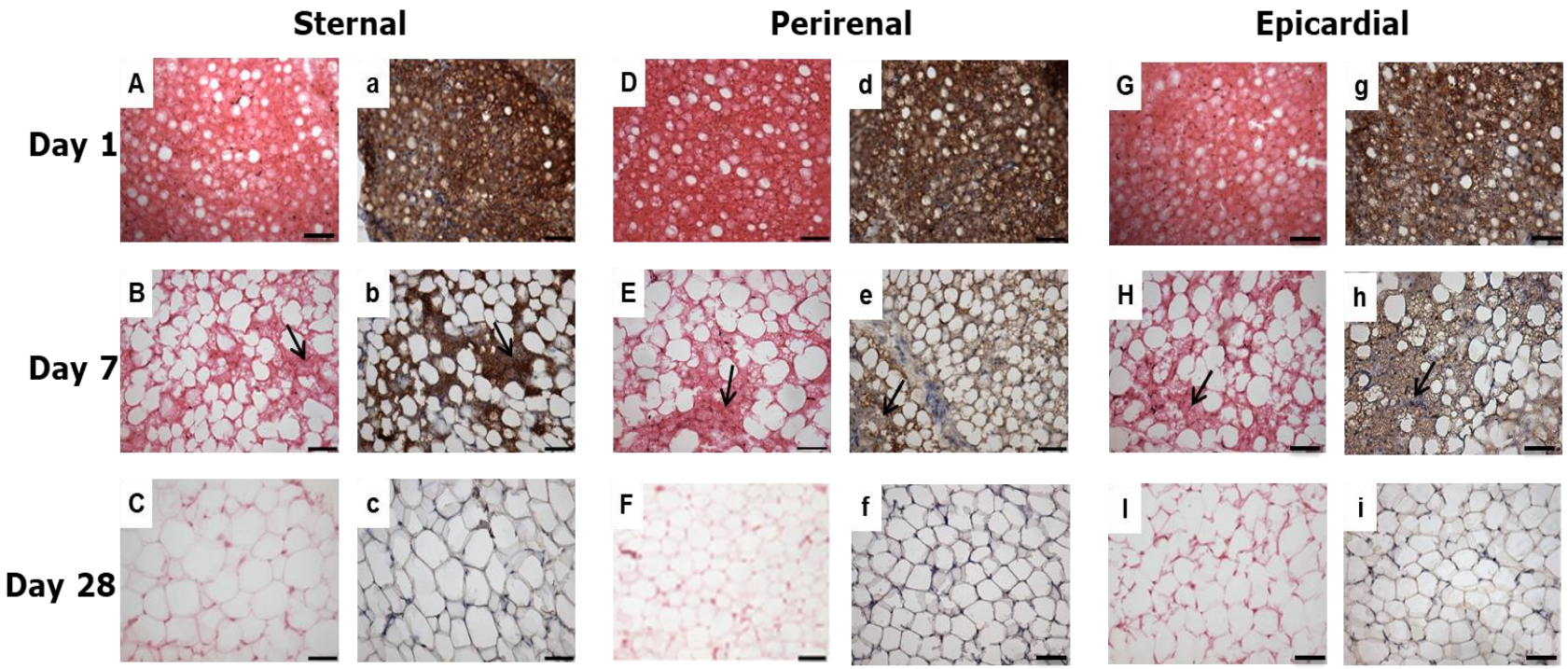



**Figure 6.4 Representative haematoxylin and eosin (H&E) and UCP1 stained microscopic histological sections from three major adipose tissue depots of lambs during their first month of age.**

Uppercase letters indicate H&E stained microscopic histological section; lowercase letters indicate UCP1 stained microscopic sections from (A-c) sternal, (D-f) perirenal and (G-i) epicardial adipose tissues. The numbers on the left indicate the age in days. The arrows indicate clusters of multilocular adipocytes, and the brown stain indicates UCP1 presence. Scale bar = 100 μm; magnification 20x.

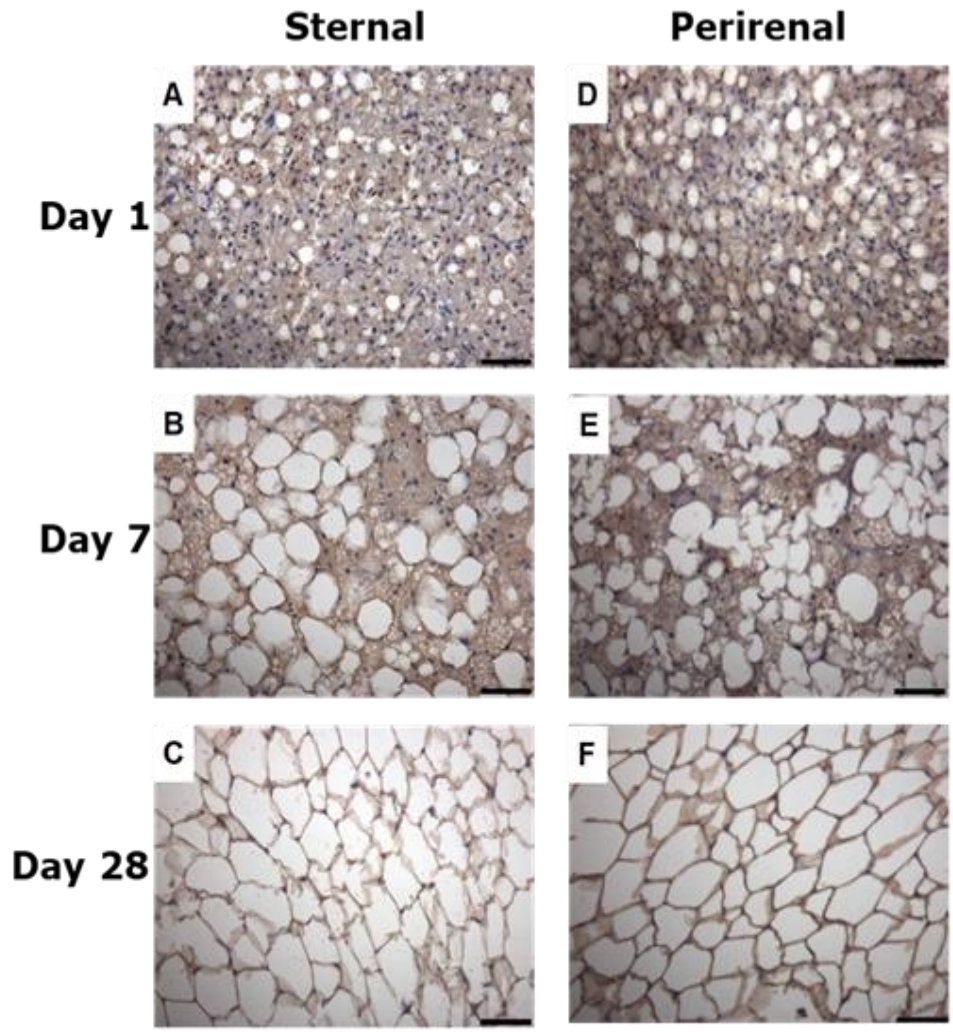



**Figure 6.5 Representative Immunohistochemistry for VDAC stained microscopic histological sections from adipose tissue depots of lambs during their first month of age.**

Uppercase letters indicate VDAC stained microscopic sections from (A-C) sternal and (D-F) perirenal adipose tissues. The numbers on the left indicate the age in days. The brown stain indicates VDAC presence. Scale bar = 100 μm; magnification 20x.

The rate of disappearance of brown adipocytes and the appearance of white space with age was different among the sternal, perirenal and epicardial depots. In the first week of age, the highest rate of appearance increase in white space was observed in the epicardial depot, with an increase of 2.4 fold ($p=0.0029$), followed closely by the sternal depot with an increase of 2.2 fold and finally the perirenal depot with an increase of 1.8 fold. In the next three weeks, the highest rate was in the sternal depot (1.8 fold) ($p=0.003$) and then in the perirenal and epicardial depots equally (1.3 fold). There were no significant differences between adipose tissue depots in each time point ($p \geq 0.05$) (Figure 6.6, Appendix 7).

White space is suggested to be an indicator of lipid droplets that occupy this area before xylene treatment. In immunohistochemistry (IHC), if the tissue is embedded in paraffin, xylene is used as a solvent of paraffin. However, xylene treatment also dissolves the lipid in depots. Therefore, stained sections will be a negative image of where the lipids occurred before IHC process and fat cells resemble large empty white circles (Tracy and Walia, 2002; 2004).

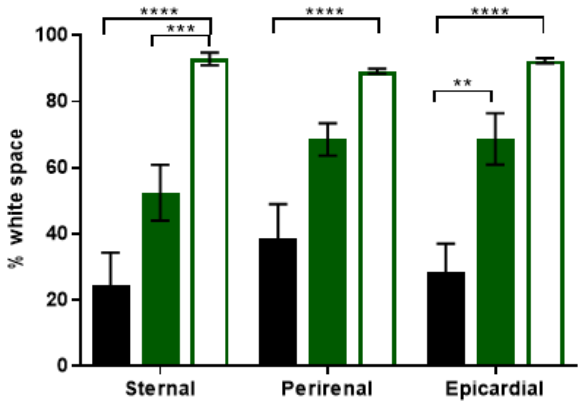



**Figure 6.6 The percentage of white space in adipose tissue depots at days 1, 7 and 28.**

Adipose tissue depots were taken from the offspring of ewes fed a standard diet. Values are mean ± SEM. Bar colour indicates sampling age: black: 1 day (n=5); green: 7 days (n=8); white: 28 days (n=8). In each time point, the adipose tissue depots were compared; and the time points in each depot were compared to each other by two-way ANOVA and Bonferroni post-hoc test. Significance is denoted by **p ≤ 0.01; ***p ≤ 0.001 and ****p ≤ 0.0001.

In conclusion, there were clear changes in the sternal, perirenal and epicardial adipose tissue appearance in early postnatal life that showed in the form of the appearance of white space in brown depots. The rate of these changes was different between adipose tissue depots, with the perirenal and epicardial adipose tissues being slower than the sternal ones in the transition to white adipose tissue (WAT) during the first month of life.

### 6.4.1.2 Changes of mitochondrial proteins abundance in adipose tissue with age

UCP1 relative abundance per mg of mitochondrial protein peaked at day 1 of postnatal age before declining significantly ($p$=0.009 and < 0.0001) by day 7 of age in both the sternal and perirenal adipose tissues, respectively. It was no longer detectable at day 28 of age (Figure 6.7 (A)). Similarly, cytochrome c and VDAC were detected in both adipose tissues, and their abundance peaked immediately after birth before gradually declining significantly (p>0.05) (Figure 6.7 (B) and (C)). Unlike UCP1, both

cytochrome c and VDAC were detectable in lamb sternal and perirenal mitochondria at day 28 of age.

In conclusion, at the early postnatal ages, some variation was observed in the abundance of these mitochondrial proteins between perirenal and sternal adipose tissue. At day 1 of age, UCP1 abundance in the perirenal adipose tissue was higher than that in the sternal adipose tissue ($p$=0.002) with no significant variations in both VDAC and cytochrome c. At day 7, only VDAC showed different abundance between the two depots with the perirenal adipose tissue having higher VDAC abundance than the sternal adipose tissue ($p$=0.032). At day 28, there was no differences between the perirenal and sternal in the abundance of UCP1 and VDAC, however, the perirenal adipose tissue had higher cytochrome c abundance than the sternal adipose tissue ($p$=0.005) (Figure 6.7).

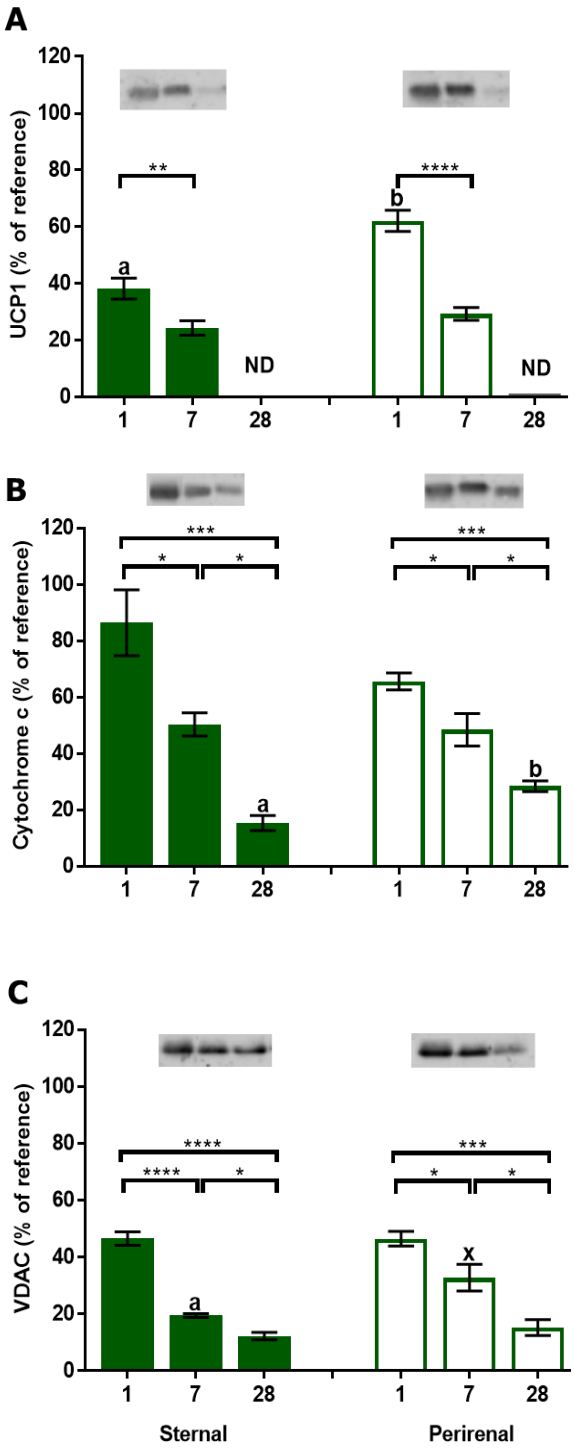



**Figure 6.7 Changes of (A) uncoupling protein (UCP)1, (B) cytochrome c and (C) voltage-dependent anion channel (VDAC) in adipose tissues at the first month of age.**

Adipose tissue depots were taken from the offspring of ewes fed a standard diet. Bar colour indicates adipose depot: green—sternal; white—perirenal. The numbers in the x-axis indicate the age of lambs in days (1: n=5, 7&28: n=8). ND; not detectable. Values are mean ± SEM. In each time point, the sternal and perirenal adipose tissue depots were compared and the time points in each depot were compared to each other by one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 and ****p ≤ 0.0001. Superscript letters denoted a significant difference between depots: a vs. b (q≤0.01), a vs. x (q≤0.05). Representative CCD-camera image of individual bands of protein are shown above the corresponding bars.

### 6.4.2 Effect of maternal nutrition on mitochondrial protein abundance in offspring adipose tissue

#### 6.4.2.1 Alterations in histological appearance of offspring adipose tissue with maternal fatty acid supplementation

To determine if maternal FA supplementation could alter the rate of loss of brown adipocytes in the offspring adipose tissue, the histology and immunohistochemistry distribution of UCP1 were repeated in the sternal, perirenal and epicardial adipose tissue depots in the three dietary groups: control, canola oil and sunflower oil supplmentation.

At day 7 of age, representative H&E and IHC images showed no clear visible differences in the number of cells stained for UCP1 in the supplemented groups compared with those in the control group in the sternal, perirenal or epicardial adipose tissues (Figure 6.8). Similarly, there were no statistically significant differences between the supplemented groups and the control group when the percentage of tissue stained for UCP1 was quantified across the three adipose tissue depots (Figure 6.10 (A)). In addition, epicardial adipose tissue showed the highest UCP1 abundance at 11% ($p$<0.0001) compared with the sternal and perirenal adipose tissue in the controls and at 13% ($p$<0.0001) compared with the supplemented groups (Table 6.1).

By day 28 of age, the abundance of UCP1 had substantially decreased (Figure 6.9). The supplemented groups' images showed small areas of multilocular adipocytes stained for UCP1 that were scattered across the three adipose tissue depots, which was not apparent in the control group. However, when the percentage of tissue stained for UCP1 was quantified, there were no significant differences between the control and supplemented groups in any of the depots except for a decline by 12% observed in the sternal adipose tissue of lambs in the sunflower group ($p$=0.0002). The epicardial adipose tissue still had a significantly higher number of UCP1 positive cells at 28 days compared with the sternal tissue, but not for the perirenal tissue, in the controls (8%, $p$=0.024) which increased to c. 19% with FA supplements

($p$=0.0001). In the perirenal adipose tissue of the controls, there was no significant difference in the abundance of UCP1 compared with the sternal adipose tissue. However, maternal FA supplementation with canola and sunflower oils resulted in increased UCP1 abundance in the perirenal tissue by 12% ($p$=0.001) and 18% ($p$=0.0001), respectively (Figure 6.10 (B), Table 6.1).

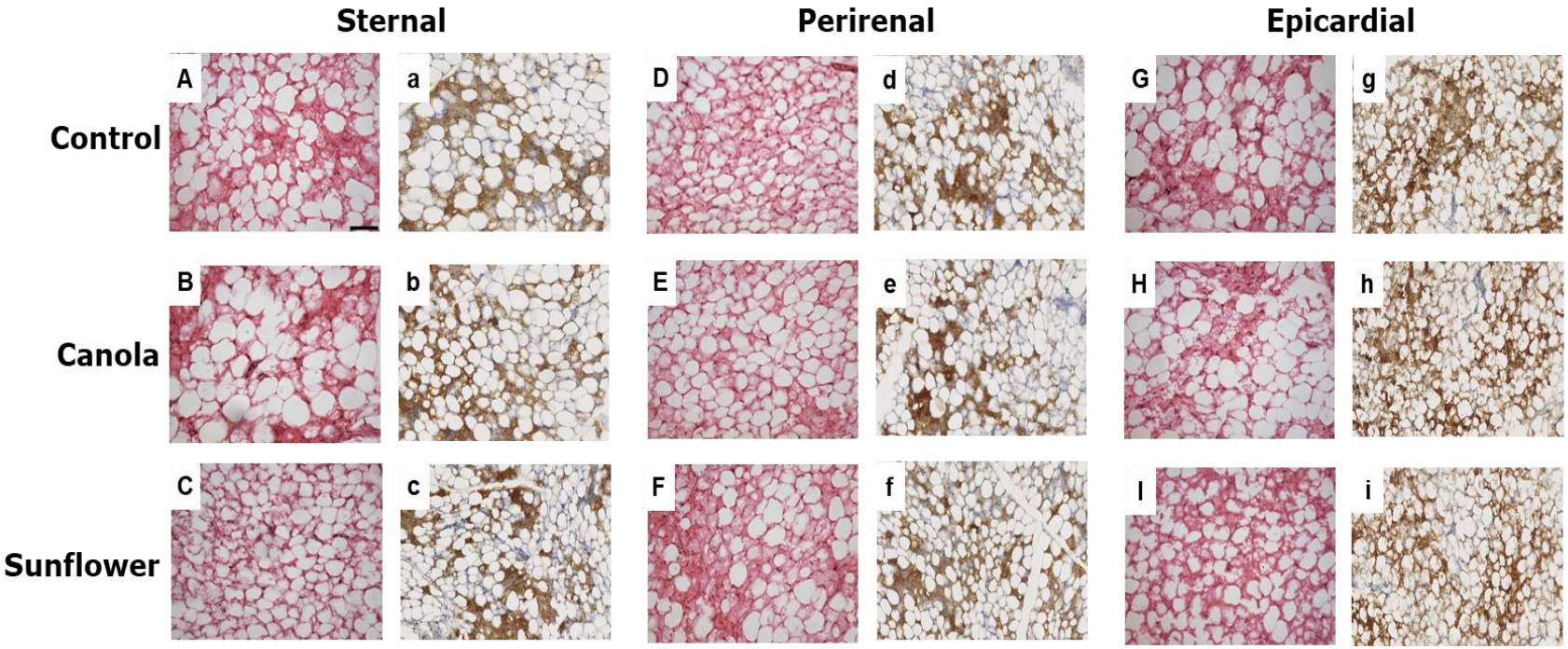



**Figure 6.8 Representative haematoxylin and eosin (H&E) and UCP1 stained microscopic histological sections from three major adipose tissue depots of lambs at day 7 of age.**

Uppercase letters indicate H&E stained the microscopic histological section; lowercase letters indicate UCP1 stained microscopic sections from (A-c) sternal, (D-f) perirenal and (G-i) epicardial adipose tissues. The dietary groups on the left relate to the three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). Brown stain indicates UCP1 presence. Scale bar = 100 μm; magnification 20x.

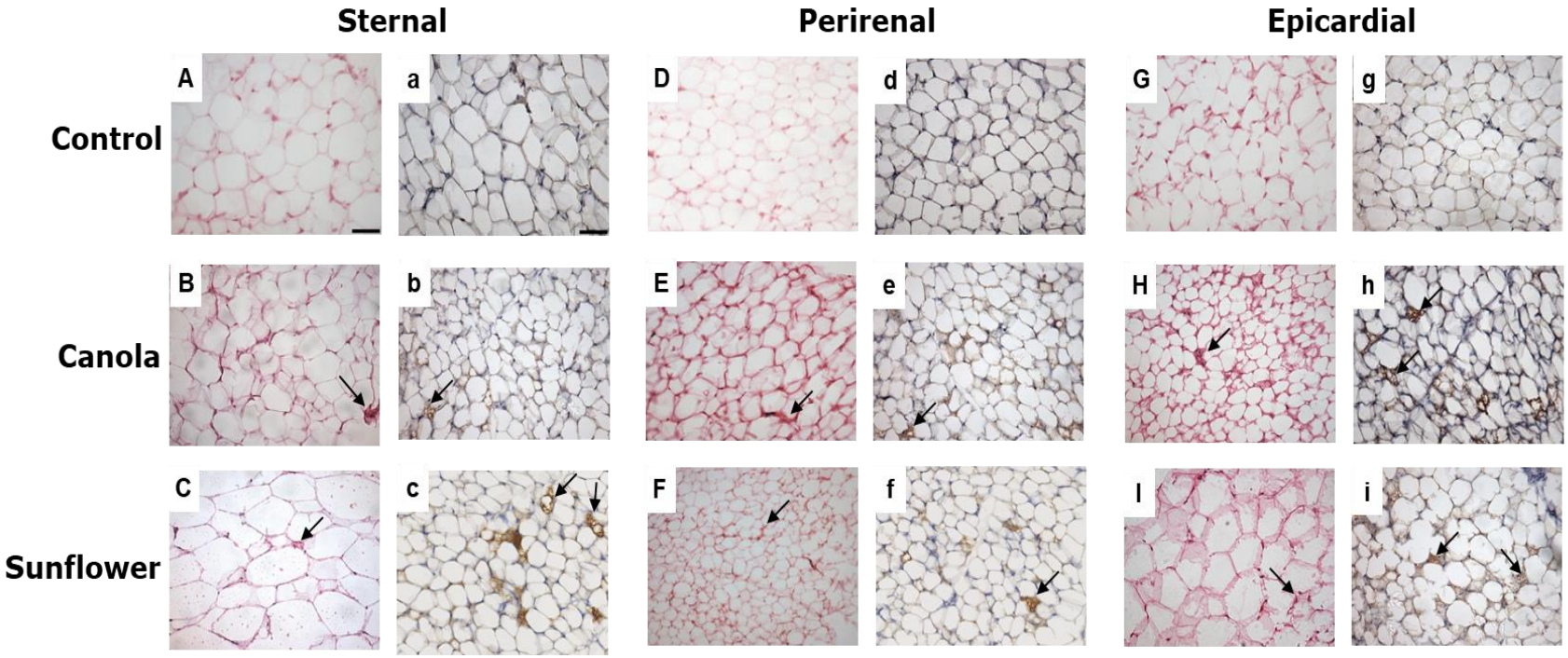



**Figure 6.9 Representative haematoxylin and eosin (H&E) and UCP1 stained microscopic histological sections from three major adipose tissue depots of lambs at day 28 of age.**

Uppercase letters indicate H&E stained microscopic histological section; lowercase letters indicate UCP1 stained microscopic sections from (A-c) sternal, (D-f) perirenal and (G-i) epicardial adipose tissues. The dietary groups on the left relate to three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). The arrows indicate clusters of multilocular adipocytes, and brown stains indicate UCP1 presence. Scale bar = 100 μm; magnification 20x.

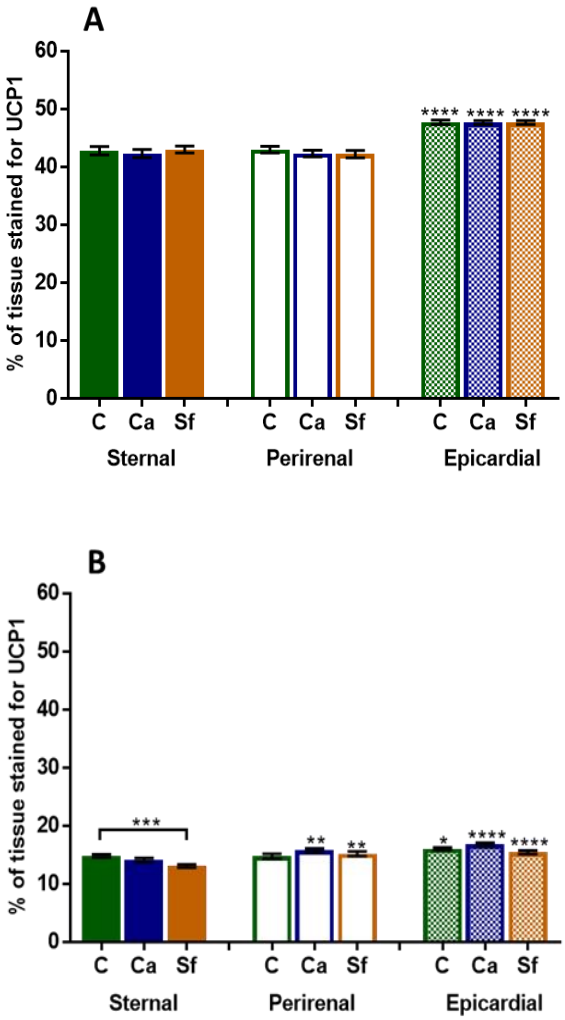



**Figure 6.10 Percentage of adipose tissue sections stained with the UCP1 antibody by immunohistochemistry in the three major adipose tissue depots at day (A) 7 and (B) 28 of age by dietary group.**

The dietary groups relate to the three maternal diets: control (C: standard diet, n=5), canola (Ca: standard diet +3% canola oil, n=5) and sunflower (Sf: standard diet +3% sunflower oil, n=5). All values are mean ± SEM. The canola and sunflower groups were compared with the corresponding control group; and within each dietary group, adipose tissue depots were compared using one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

**Table 6.1 Comparison of the percentage of adipose tissues stained with the UCP1 antibody between the major adipose tissue depots at days 7 and 28 of age by dietary groups.**

| | Control (n=5) | | Canola (n=8) | | Sunflower (n=8) | |
|---|---|---|---|---|---|---|
| | Day 7 | Day 28 | Day 7 | Day 28 | Day 7 | Day 28 |
| Perirenal vs. Sternal | NS | NS | NS | ⇧12% P=0.001 | NS | ⇧18% P=0.0001 |
| Epicardila vs. Sternal | ↑11% p<0.0001 | ↑ 8% p=0.024 | ↑13% p<0.0001 | ↑19% p<0.0001 | ↑11% p<0.0001 | ↑18% p<0.0001 |
| Epicardila vs. Perirenal | ↑11% p<0.0001 | ↑ 9% P=0.015 | ↑13% p<0.0001 | NS | ↑13% p<0.0001 | NS |

The dietary groups relate to the three maternal diets: control (standard diet), canola (standard diet +3% canola oil) and sunflower (standard diet +3% sunflower oil). All values are the relative change between the depots. Adipose tissue depots were compared using one-way ANOVA and Bonferroni post-hoc test. Directional arrows denote significant differences. NS = not significant ($p \geq 0.05$).

#### 6.4.2.1.1 Adipocyte size

Adipocyte sizes were measured at days 7 and 28 of age for sternal, perirenal and epicardial adipose tissue samples (Figure 6.11)

At day 7, there was a significant decrease in adipocyte size in both the sternal and epicardial depots in the sunflower group compared with the control group ($p$=0.0001 and $p$=0.0004, respectively). In contrast, perirenal adipocyte size increased by 27% in the canola group and 22% in the sunflower group compared with controls ($p$=0.0001) (Figure 6.11 (A)).

By day 28, however, the size of perirenal adipocytes had decreased significantly in both the sunflower and canola groups compared with the control group by 34% and 12% ($p$=0.0001, $p$=0.002), respectively. Furthermore, a significant increase in the size of sternal adipocytes and, to a lesser extent, epicardial adipocytes by 63% and 19% ($p$=0.0001), respectively, was observed in the sunflower group compared with the control group (Figure 6.11 (B)).

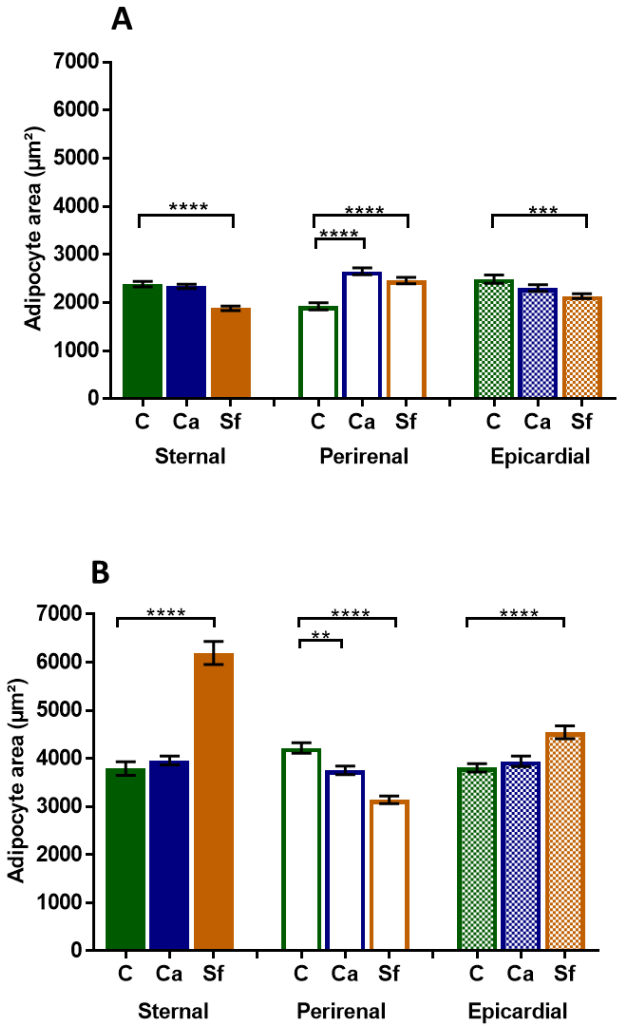



**Figure 6.11 Mean white adipocyte size (µm²) of major adipose tissue adipocytes at day (A) 7 and (B) 28 of age.**

The dietary groups relate to the three maternal diets: control (C: standard diet, n=5), canola (Ca: standard diet +3% canola oil, n=5) and sunflower (Sf: standard diet +3% sunflower oil, n=5). All values are mean ± SEM. The canola and sunflower groups were compared with the corresponding control group using one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

#### 6.4.2.1.2 Adipocyte count

The mean of the total number of adipocytes within the same area of each adipose depot in all dietary groups at days 7 and 28 of age are shown in Figure 6.12.

The same pattern of increased cell count was seen across all adipose tissue depots in the three dietary groups at day 7 of age, where the largest number of cells was in the sunflower group, followed by the canola and control groups. However, the canola oil supplement had no significant effects on the cell count across all adipose tissue depots, while the cell count increased significantly, by 86%, in both the perirenal ($p$=0.002) and epicardial adipose tissue depots ($p$=0.027) with the sunflower supplement when compared with the controls (Figure 6.12 (A)).

At day 28 of age, there was a wide inter-group variation in cell counts across the dietary groups, and there appeared to be a higher number of cells in controls compared with the supplemented groups, but this was not statistically significant for either the sternal or epicardial adipose tissues. In contrast, in the perirenal adipose depot, the number of cells doubled in the sunflower group ($p$=0.0074) compared with the control group, while no significant difference was seen in the canola group (Figure 6.12 (B)).

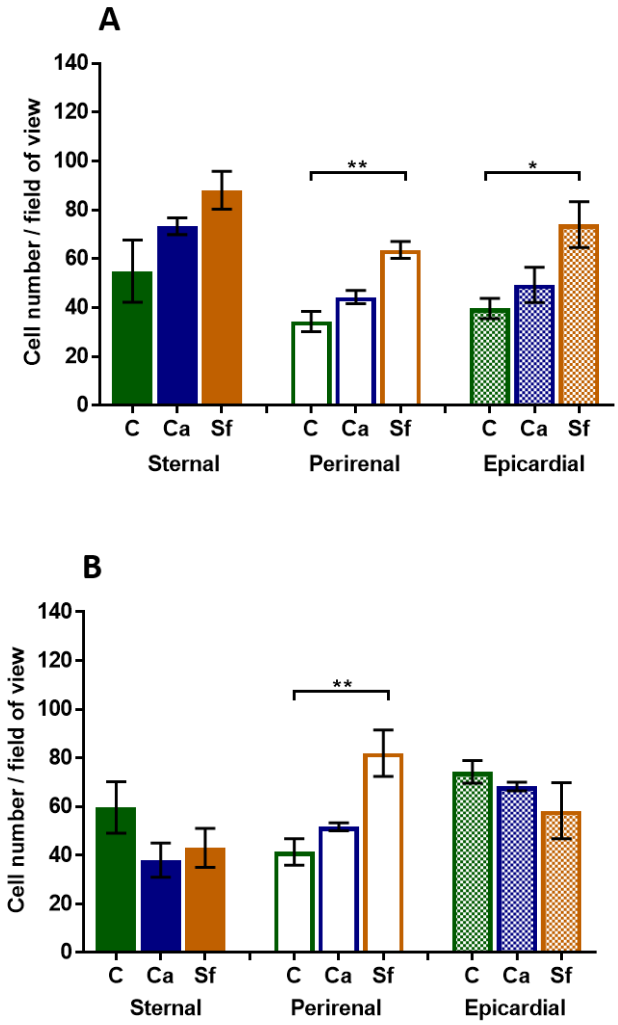



**Figure 6.12 Mean white adipocyte count of adipocytes from major adipose tissue depots at day (A) 7 and (B) 28 of age.**

The dietary groups relate to the three maternal diets: control (C: standard diet, n=5), canola (Ca: standard diet +3% canola oil, n=5) and sunflower (Sf: standard diet +3% sunflower oil, n=5). All values are mean ± SEM. The canola and sunflower groups were compared with the corresponding control group using one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *$p \leq 0.05$; **$p \leq 0.01$.

### 6.4.2.2 Effect of maternal fatty acid supplementation on offspring adipose tissue mitochondrial protein abundance

After finding adipocytes positively stained with UCP1 antibody at day 28 of age in adipose tissues of lambs fed by supplemented mothers compared with controls, a Western blot analysis on the sternal and perirenal adipose tissue samples was performed

to obtain another quantifiable measure of UCP1, as well as other mitochondrial, protein abundance.

There was a significant increase in mitochondrial protein abundance in both the sternal and perirenal adipose tissue depots with canola oil supplements at both day 7 and 28 of age. At day 7, compared with the controls, UCP1 increased by 57% in the sternal ($p$=0.015) and by 48% in the perirenal adipose depot ($p$=0.001) (Figure 6.13 (A)). Cytochrome c abundance increased 6 fold in the sternal ($p$=0.006) and 7 fold in the perirenal tissue sample (0.002) (Figure 6.13 (B)). Although the canola oil supplements did not affect VDAC abundance in the sternal tissue, VDAC abundance increased by 64% in the perirenal tissue samples ($p$=0.05) (Figure 6.13 (C)). At this time point, there was no significant differences between the depots in UCP1 abundance in each dietary group; however, cytochrome c was more abundant in the sternal depot by 2 fold ($p$=0.019) in the sunflower group while VDAC was more abundant in the perirenal depot by 68% ($p$=0.032) in the controls.

At day 28, UCP1 was detected in the perirenal adipose depot of lambs for the canola, and to lesser extent sunflower, supplemented groups, with a relative abundance per mg mitochondrial protein of 8% ($p$=0.043) and 2%, respectively (Figure 6.14 (A)). Similar to 7 days of age, cytochrome c abundance remained 6 fold more in the sternal ($p$=0.007) but only 3 fold more in the perirenal ($p$=0.003) depot in the canola oil supplemented group when compared with controls (Figure 6.14 (B)). The pattern of the increase of VDAC in the supplemented groups compared with controls were similar in both sternal and perirenal adipose tissue depots, with an increase of 1.5 fold and c. 60%, respectively (Figure 6.14 (C)). At this time point, the perirenal tissue showed more UCP1 abundance with canola oil supplements and in cytochrome c in controls than the sternal adipose depot, with no difference between the depots regarding VDAC abundance.

In summary, maternal supplementation with canola oil increased the abundance of UCP1 in offspring perirenal adipose tissue. Moreover, canola oil, and to a lesser extent sunflower oil, supplements increased the abundance of cytochrome c and VDAC in both perirenal and sternal adipose tissue depots compared with the controls.

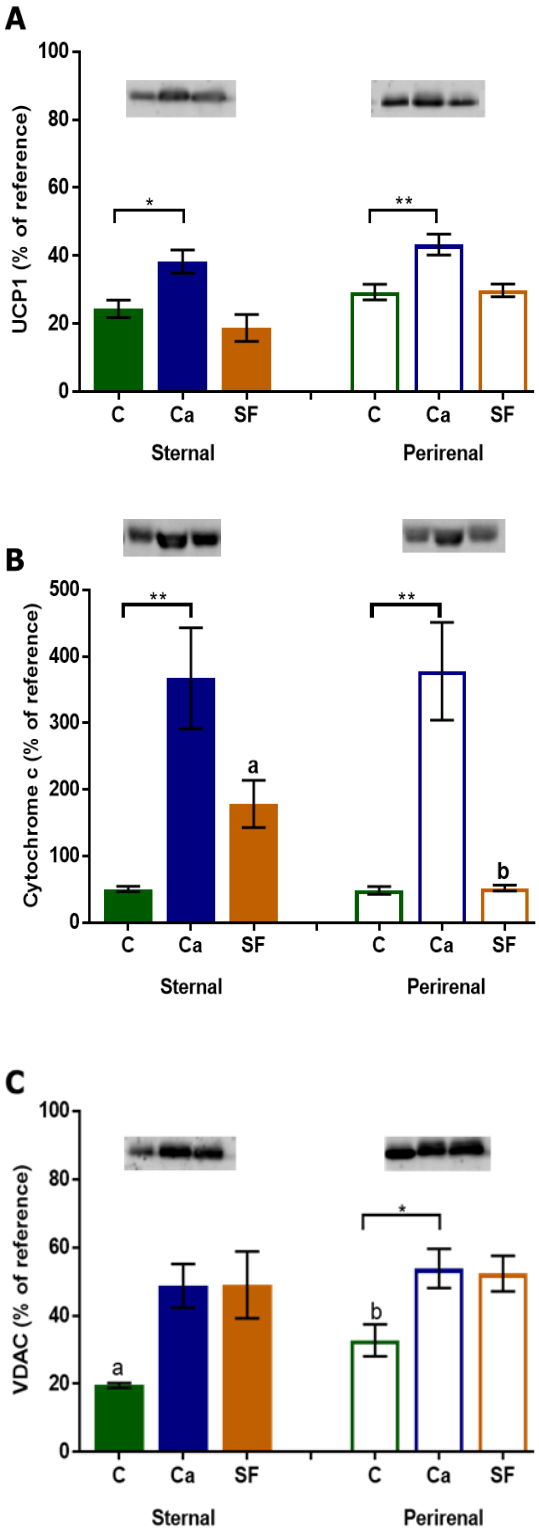



**Figure 6.13 Changes of (A) uncoupling protein (UCP)1, (B) cytochrome c and (C) voltage-dependent anion channel (VDAC) in adipose tissues at day 7.**

The dietary groups relate to the three maternal diets: control (C: standard diet, n=8), canola (Ca: standard diet +3% canola oil, n=8) and sunflower (SF: standard diet +3% sunflower oil, n=8). All values are mean ± SEM. The canola and sunflower groups were compared with the corresponding control group; and for each dietary group, sternal and perirenal adipose tissues were compared using one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *p≤0.05, ** p≤0.01. Superscript letters denoted a significant difference between depots: a vs. b (p≤0.05). Representative CCD-camera image of individual bands of protein are shown above the corresponding bars.

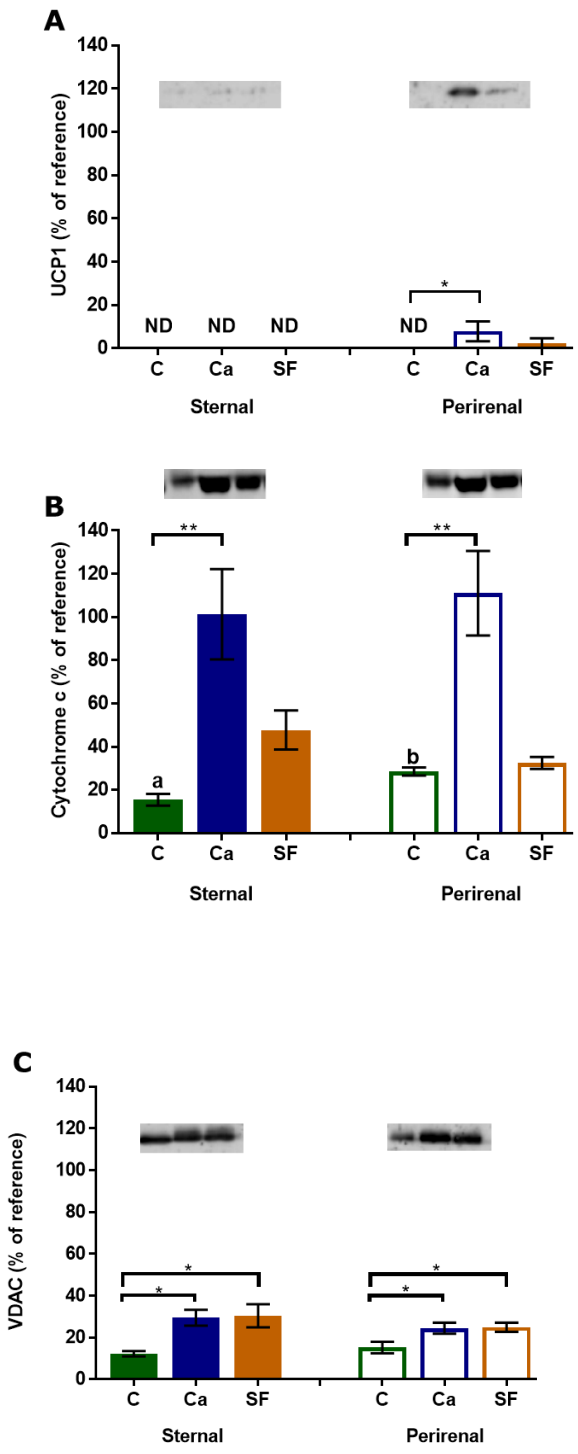



**Figure 6.14 Changes of (A) uncoupling protein (UCP)1, (B) cytochrome c and (C) voltage-dependent anion channel (VDAC) in adipose tissues at day 28.**

The dietary groups relate to the three maternal diets: control (C: standard diet, n=5), canola (Ca: standard diet +3% canola oil, n=8) and sunflower (SF: standard diet +3% sunflower oil, n=8). ND = not detectable. All values are mean ± SEM. The canola and sunflower groups were compared with the corresponding control group; and for each dietary group, sternal and perirenal adipose tissues were compared using one-way ANOVA and Bonferroni post-hoc test. Significance is denoted by *p≤0.05, ** p≤0.01. Superscript letters denoted a significant difference between depots: a vs. b (p≤0.05). Representative CCD-camera image of individual bands of protein are shown above the corresponding bars.

## 6.5 Discussion

Brown adipose tissues are distinguished by their large amounts of UCP1, which when activated, allow the free flow of protons across the inner mitochondrial membrane, producing heat because of the rapid dissipation of chemical energy (Cannon & Nedergaard, 2004). In sheep, brown adipose tissues gradually become white as age increases, which is accompanied with the loss of UCP1 (Clarke et al., 1997). However, the difference between adipose tissue depots in such transformation is still largely unexplored in large mammals.

The present study endorsed the established differences among adipose tissue depots during early life of sheep. Along with this, it also revealed that the rate of appearance of white spaces, an indicator of lipid droplets which were occupied these areas, is different between each adipose depot examined (e.g., sternal, perirenal and epicardial). The rate of transition from being a brown depot to a depot populated with white adipocytes is higher in the sternal than in epicardial and perirenal adipose tissues.

UCP1, VDAC and cytochrome c play a key role in cellular energy regulation, particularly during the transition from foetal to neonatal life (Mostyn et al., 2004). In this phase, the requirements of energy are almost at their maximum rates (Hillman et al., 2012). Mostyn et al. (2003) reported that the abundance of UCP1, VDAC and cytochrome c proteins in the perirenal adipose tissues peaked on day 1 of age. Then, the abundance of VDAC and cytochrome c declined significantly by day 7 of age, continuing up to day 30 of age. By the end of the first month, UCP1 could not be detected. The data obtained from the control group in the current investigation are in agreement with Mostyn et al. (2003). The parallel decline in UCP1, VDAC and cytochrome c as postnatal age increases was not unexpected because the whole-body metabolic rate similarly declines over this period (Symonds et al., 1989).

Interestingly, the abundance of these mitochondrial proteins varied between the perirenal and sternal adipose tissue depots, with the former having more UCP1 at day 1, more VDAC at day 7 and more cytochrome c at day 28. These results are in line with

Chapter 5 results, which suggested a higher metabolic activity in the perirenal adipose tissue depot compared to the other adipose tissue depots. These findings were supported by those of Holloway et al. (1985). They reported an association between non-shivering thermogenesis in the perirenal adipose tissue of adult dogs with an increase of mitochondrial GDP-binding and cytochrome oxidase activity.

Importantly, this adaptive loss of the mitochondrial proteins in both the perirenal and sternal adipose tissues was markedly delayed in lambs fed by ewes receiving FA supplementation during lactation. The quantification of adipose tissue mitochondrial proteins by Western Blotting obtained from the control, canola oil supplemented and sunflower oil supplemented groups suggested that maternal supplementation with canola oil can increase the abundance of UCP1 in offspring perirenal adipose tissue. Moreover, canola oil, and to some extent sunflower oil, supplements increased the abundance of cytochrome c and VDAC in both perirenal and sternal adipose tissue depots. Both of cytochrome c and VDAC have been reported to be effected by diet (Yakubu et al., 2007). Therefore, it is possible to assume that the oxidation of FAs in canola oil occurs through increased involvement of UCP1 in the perirenal adipose depot (Fedorenko et al., 2012) while this is not the case in the sternal adipose depot. On the other hand, sunflower oil supplements up-regulated FA oxidation (Van Hinsbergh et al., 1980) by enhancing the involvement of cytochrome c which in turn utilises VDAC for selective oxidation of membrane-permeable respiratory substrates such as short-chain FAs (Lemasters & Holmuhamedov, 2006).

Cytochrome c abundance in the canola oil supplement group was much higher than control group in both sternal and perirenal adipose tissue depots at both day 7 and 28 of age with no such effect of sunflower oil supplement at both time points. In ovine, the abundance of cytochrome c was vary between lung, kidney and liver tissues obtained from fetuses  and offspring with different tissue specific responses to changes in maternal diet (Yakubu et al., 2007). Maternal nutrient restriction between early to mid gestation resulted in increased cytochrome c abundance at 140 days gestation in the

liver, 1 day of age in the kidney and 6 months of age in lungs (Yakubu et al., 2007). It has been reported that dietary fat manipulations are able to alter the activities of some key metabolic enzymes in the mitochondrial electron transport chain such as ubiquinol-2-cytochrome c reductase (complex III) (Barzanti et al. 1994) and cytochrome oxidase (complex IV) (Barzanti et al. 1994; Yamaoka et al. 1988). Barzanti et al. (1994) found changes in the activities of these enzymes in the mitochondria of liver, heart and brain of rats fed on diets differing in their polyunsaturated fatty acid (PUFA) compositions. Several lipids have been proposed as playing a crucial role in mitochondrial membrane enzymatic activity (Gonzalvez & Gottlieb 2007; Osman et al. 2011). Monteiro et al. (2013) reported differences in liver mitochondrial membrane composition and function after feeding the rats diet containing 20% rapeseed oil and were attributed the effects to the erucic acid, the major component in rapeseed oil (Abdellatif & Vles, 1970). Alterations in mitochondrial membrane lipid composition could be an adaptation mechanism to face changes in the FAs available for de novo synthesis, in order to maintain sufficient membrane physical properties (Innis & Clandinin, 1981). Although Monteiro et al. (2013) reported no differences between rapeseed oil supplemented groups and controls in the expression of several mitochondrial protein including cytochrome c, it remains to be investigated whether a longer-term supplementation would contribute to an alteration in the mitochondrial protein abundance to naturalise changes in mitochondrial activity that induced by lipid. An increase in cytochrome oxidase level resulted in increased cytochrome c in the mitochondria from camptothecin-treated Jurkat cells (Sanchez-Alcazar et al, 2000). Although adipose tissue mitochondrial respiration and lipid composition were not investigated in the present study, it could be assumed that the increase seen in cytochrome c abundance in adipose tissue samples from the canola oil supplemented group was mediated by erucic acid in milk (Monteiro et al., 2013). As shown in Chapter 3 (Section 3.4.1.3), there was an increase of erucic acid content by 33% ($q$= 0.01) in the milk obtained from ewes fed a diet supplemented with canola oil. However, it should be emphasised that it is not possible to attribute any observation in my investigation to any single FA present in the milk. Further

investigation into the role of canola oil erucic acid in increasing cytochrome c abundance or activity may potentially be of research interest. However, pursuing erucic acid as a potential therapeutic intervention would not be approrpiate as it has been reported to be associated with negative effects on cardiac health (Aherne et al., 1976; Beare-Rogers et al., 1974; Bozcali et al., 2009; Hung et al., 1977). As previously mentioned in Chapter 3; Section 3.5.3.1, it should be noted that erucic acid content in milk samples obtained in this investigation not exceed a tolerable level of human exposure (FSANZ, 2003).

Brown adipose tissues have an important role in preventing hypothermia in newborn sheep through UCP1 activation (Henry et al., 2017). In relation to the effect of the mother's diet on offspring thermogenic capacity, Ojha et al. (2013) found that the expression of most of the BAT-related genes decrease along with UCP1 during early life in neonatal sheep born to nutrient-restricted ewes, while genes related to WAT remain unaffected. Despite the current findings showing a significant decrease of mitochondrial protein abundance with age, no significant effect of maternal supplementation with canola oil was found on the abundance of UCP1 in adipose tissue depots at either day 7 or 28 of age. Interestingly, when data of the three adipose tissue depots were subjected to comparison, maternal FA supplementation with either canola or sunflower oil were shown to have differential effects on the percentage of adipose tissues stained with the UCP1 antibody. The comparison of UCP1 abundance between adipose tissue depots showed that epicardial adipose tissue has more UCP1 than sternal and perirenal in all dietary groups at both time points (except the perirenal in supplemented groups at age of 28 days). It has been considered that adipose tissues are metabolic organs with important functions beyond lipid storage (Henry et al., 2017). Nevertheless, the extent to which these functions vary between adipose tissue depots is largely unexplored, especially during early life development. To the best of my knowledge, this is the first time that canola or sunflower oil supplementation can increase UCP1 in epicardial adipose tissue, compared with sternal depots, has been reported. My finding supports the concept that adipose tissue functions as several autonomic organs that seem to have

distinct functions (Henry et al., 2017; Macotela et al., 2012). In addition, these results are in agreement with an Ojha et al. (2016) study in children, in which they reported a relatively large number of brown adipocytes in the epicardial adipose tissue depot in newborns, extending until the infancy period. The high abundance of UCP1 in epicardial adipose tissue, compared with the other two adipose depots, could be a result of special properties of epicardial adipocytes to manage FAs in order to maintain myocardial physiology including high rates of FA incorporation, FA synthesis or FA breakdown (Rabkin, 2007).

Adipose tissue grows by two mechanisms: increasing the cell size through 'hypertrophy' and increasing the cell number through 'hyperplasia' (Jo et al., 2009). Because of its important storage capacity, adipocytes are likely to change in volume in greater amplitudes (Farnier et al., 2003). In humans, adipocyte size may increase up to 3 fold (Ginsberg-Fellner & Knittle, 1981). In obese subjects, several metabolic functions of adipose tissue are subject to change with adipocyte size, including storage and the mobilisation of lipids in humans (Jamdar and Osborne, 1981) and rats (Zinder and Shapiro, 1971). Classically, the number of adipocytes throughout a life cycle is considered to stay constant (Berry et al., 2014). However, there are reports suggesting that the number of adipocytes varies with age and diet (Bertrand et al., 1978; Fischer-Posovszky et al., 2010). In the present study, compared with the controls, a significant decrease in cell size in both sternal and epicardial adipocytes were observed at day 7 of age in the sunflower oil supplemented group while perirenal adipocyte size increased significantly with both canola and sunflower oils. On the contrary, at day 28 of age, adipocyte size in the perirenal adipose depot decreased significantly in the sunflower, and to a lesser extent in canola, oil supplemented offspring, while sternal and epicardial adipocytes significantly increased in size in the sunflower supplemented group. A decrease in adipocyte size is accompanied with FA oxidation and thermogenesis (reviewed in Choe et al., 2016), whereas the increase of size could be associated with the storage of fat (MacLean et al., 2015). Consequently, supplementation with sunflower

oil may have enhanced the energy consumption by FA oxidation in sternal and epicardial adipose depots at day 7 of age, whereas canola oil did the same only in the perirenal adipose depot at day 28 of age. Regarding the number of adipocytes, the cell count increased by day 28 of age in the perirenal adipose tissue depots with the sunflower oil supplement compared with the controls. An increase in the number of adipocytes could be related with WAT expansion and adipogenesis (Jang et al., 2016).

### 6.5.1 Variable results between the expression of UCP1 mRNA and protein

The UCP1 protein abundance results assessed by Western blotting were not consistent with the mRNA expression results, specifically in the perirenal adipose tissue of lambs in the canola oil supplement group. The mRNA expression data (Chapter 4, Section 4.4.5.1) did not show any effect of maternal FA supplementation on UCP1 gene expression among the offspring's perirenal, epicardial, or sternal adipose tissue depots compared with controls. In contrast, Western blotting showed that maternal canola oil supplements increased the abundance of UCP1 in their offspring's perirenal adipose tissue but not in sternal adipose tissue depots. This finding is supported by Nagano et al.'s (2015) view that perirenal adipose tissue depot is closely associated with PRDM16-EHMT1 complex expression. Additionally, PRDM16 facilitates preinitiation complex (PIC) formation and consequently, UCP1 transcription (Seale, 2015). Furthermore, since the microarray results showed that the perirenal adipose tissue depot is the main site of thermogenisis –related genes (Chapter 5, Section 5.4.1) and hence, thermogenesis, it was not surprising to see an increase in the abundance of UCP1 protein in the perirenal adipose tissue depot.

The difference between UCP1 protein abundance and mRNA expression has not been explained directly by literatures. It is generally considered that the expression of proteins is a function of mRNA expression (Guo et al., 2008). However, at a given point in time, the availability of both proteins and mRNA species depends on their life and stability in the cellular environment (Hargrove & Schmidt, 1989). For example, Futcher

et al. (1999) found a strong correlation between protein abundance and mRNA abundance in yeast. In contrast, a study of liver samples found a correlation coefficient of 0.48 between mRNA and protein abundance (Anderson & Seilhamer, 1997). Researchers have proposed many potential reasons for these weak correlations such as (i) cellular control systems cannot be observed in mRNA as they operate entirely in the protein domain (Johnston et al., 1997); (ii) protein is more stable than mRNA in most tissues (Johnston et al., 1997); and (iii) UCP1 mRNA has been reported to degrade at a faster rate than proteins (Gospodarska et al., 2015).

In addition, a role for the IGF2BP2/IMP2 protein complex's in down-regulating UCP1 cannot be ruled out (Dai et al., 2015). Dai et al. (2015) demonstrated that IGF2BP2/IMP2-deficient mice express higher levels of UCP1 mRNA and remain lean throughout life due to the high expression of UCP1. They showed that IMP2 binds the mRNAs encoding UCP1; hence, the absence of IMP2 results in more UCP1 mRNA. In vitro, the same study revealed that IMP2 inhibits the translation of mRNAs bearing UCP1 untranslated segments. An association between IGF2BP2/IMP2 and obesity and type 2 diabetes has been reported (Frau et al., 2017). Despite the lack of ample literature on the effect of supplementing the diet with PUFA or vegetable oils on the expression of IGF2BP2/IMP2 in adipose tissues, Simon et al. (2014) reported an overexpression of IGF2BP2/IMP2 in mice after dietary supplementation with methionine and choline. Although IGF2BP2 gene expression was not tested in the present study, analysing the microarray data revealed an upregulation of IGF2BP2 expression upon maternal supplementation with canola oil, and the highest expression was seen in epicardial adipose tissue depots followed by perirenal and sternal depots (data shown in Appendix 6).

In conclusion, in light of the microarray data supported by the above suggestions and findings (Anderson et al., 1998; Dai et al., 2015; Frau et al., 2017), it is possible to assume that the increased UCP1 protein abundance which was not accompanied by an increase in the relative mRNA expression in perirenal adipose tissue depots in the canola

oil supplemented group is due to a weaker correlation coefficient between protein and mRNA along with the upregulation of IGF2BP2/IMP2 compared with the control group. Finally, as both of mRNA and protein were measured in this study, it can be considered that protein is the more reliable measure (Laurent et al., 2010).

### 6.5.2 UCP1 gene expression corresponds well with adipose tissue depots morphology

Overall, there was a consistency between UCP1 mRNA expression results (Chapter 4, Section 4.4.5.1) and quantification results in the three adipose depots across the dietary groups at both time points. In general, there was no effect of maternal supplementation on UCP1 expression in all depots with epicardial adipose depot has more UCP1 than sternal and perirenal. However, there was a slight decrease in UCP1 quantity seen in the sternal adipose depot at day 28 in the lambs of ewes supplemented with sunflower oil (Section 6.4.2.1). It is possible that this occurred because the fragments used for histology and IHC were cut from samples fixed in formalin, whilst the fragments used for mRNA extraction were cut from separate frozen samples. Thus, it is likely that the samples could have been taken from different parts of the excised sternal adipose depot, since there are two depots on each side of the neck. As previously discussed, a single adipose depot could contain different cell types that vary in their responses. Furthermore, sternal adipose tissue contains lymph nodes, which, although they were removed after dissection, may have had an effect on sternal gene expression and/or tissue morphology, as some interaction between lymph nodes and the surrounding adipose tissue has been reported (Pond & Mattacks, 1995).

## 6.6 Conclusion

In adipose tissue depots, the abundance of UCP1, VDAC and cytochrome c peaked on the first day of age before subsequently decreasing with age. Although VDAC and cytochrome c abundance were closely correlated with the loss of UCP1, they were present in adipose tissue depots when UCP1 was undetectable (i.e., 28 days of age).

Furthermore, the present study has demonstrated that there is a marked divergence in the abundance of these mitochondrial proteins between adipose tissue depots, regardless of the diet.

Maternal FA supplementation during lactation with canola oil, and to a lesser extent sunflower oil, resulted in an increase of the abundance of these mitochondrial protein abundance, especially regarding UCP1 being detected in perirenal adipose depots at day 28 of age. Parallel changes in these mitochondrial proteins in lamb adipose tissue suggests their importance in maintaining BAT thermogenesis in early life. In addition, these changes coincides with the transition of adipose tissue depots from brown to white. Interestingly, the rate of change in UCP1, VDAC and cytochrome c within perirenal adipose tissue depots can be significantly altered by maternal canola oil supplementation during lactation.

# Chapter 7. Conclusion

## 7.1 General aims

The studies detailed in my thesis aimed to determine whether maternal fatty acid supplementation with a readily available short-chain fatty acid (i.e. canola or sunflower oil) modifies the milk fatty acid profile and, thus, the development of the adipose tissue in offspring. This was achieved by assessing sheep adipose tissue depots weight, histological appearance and the abundance of UCP1 and other mitochondrial proteins. Furthermore, this study focused on the mRNA expression of regulatory genes associated with thermogenesis and fatty acid metabolism. Interestingly, a more comprehensive understanding of the influence of anatomical location on adipose tissue's cellular composition and development was achieved by analysing large numbers of differentially expressed genes from microarray data. The recent increases in computer power, improvement of computational strategies and accumulation of transcriptomic data helped to express genetic relationships in a quantifiable fashion for building prediction models. These models were formed by identifying clustered genes with similar co-expression regulation and which are involved in similar and/or interdependent biological functions. Variations in the transcriptome with maternal fatty acid supplementation between similar tissues could reflect the differences in adipocyte type, function, transcriptional programme and cellular composition. This allowed interpretation of differential network analyses and taking advantage of the results with an improved understanding of adipose tissue development.

This chapter summarises the key findings and main limitations of these studies and investigations.

## 7.2 Methods

Ewes rearing twin lambs were fed a control diet or one supplemented with 3% canola or with 3% sunflower oil from the day of delivery throughout lactation in order to alter the

fatty acid profile of their milk. Milk samples and ewe and lamb weights were taken at 7 and 28 days, and offspring underwent tissue and blood sampling at either 7 or 28 days of age. The mRNA expression of regulatory genes associated with thermogenesis and fatty acid metabolism was measured. The abundance of UCP1 and other mitochondrial proteins was determined by immunohistochemistry and immunoblotting. Finally, microarray datasets were subjected to multi-region bioinformatic analyses in order to obtain biologically meaningful information about the examined adipose tissue depots.

## 7.3 Summary of findings

A summary of the research methods, hypotheses and main results is shown in Figure 7.1.

### 7.3.1 Manipulating dietary fatty acid supplementation modulates milk fatty acid profiles

In agreement with previously published studies (Hervás et al., 2008; Okine et al., 2003; Toral et al., 2010; Welter et al., 2016), canola/sunflower oil dietary supplements resulted in pronounced changes in milk saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and *trans* fatty acids (TFAs) throughout the first month of lactation. Both supplements decreased the milk content of medium-chain SFAs and long-chain SFAs up to C16:0, all be it at the expense of an increase in C18:0. Neither supplement had an effect on ω-3 fatty acids. Milk MUFAs increased more with canola oil than they did with sunflower oil. An opposite effect of canola and sunflower oils was seen on milk ω-6; the former reducing the majority of these acids, while the latter increased LA, and, thus, increased total ω-6 fatty acids. Despite the changes in the milk fatty acid profiles induced by supplementation, large increases in TFAs were also observed. It is noteworthy that TFA concentrations measured in this study are far below the upper limit in infant formula as determined by the European Food Safety Authority (EFSA, 2006).

### 7.3.2 Maternal sunflower oil supplementation increased adiposity in female offspring

Maternal supplementation with sunflower oil increased female offspring adipose tissue weights and growth rates but not total body weights. This could be due to the increase of ω-6 LA in the milk received by these lambs (Javadi et al., 2004; Weiler, 2000). The differential effect of gender on adipose tissue mass requires further investigation in order to be confirmed and to determine potential mechanisms.

### 7.3.3 Maternal fatty acid supplementation affects expression pattern of genes associated with fatty acid metabolism across adipose tissue depots

A set of genes associated with fatty acid metabolism (i.e. β Oxidation, fatty acid synthesis and storage) were studied to gain more comprehensive insight into the effect of maternal supplementation with fatty acids during the early life of sheep. All the examined genes showed quantitative differences in gene expression among the three depots, which were low in numerical value but statistically significant; the highest and the lowest gene expression being seen in the epicardial and sternal adipose tissue depots respectively. Although the observed expression patterns of the most examined genes were not significantly affected by the fatty acid supplements, the groups receiving supplements had slightly more variation in gene expression profiles between adipose tissue depots compared with depots in controls. The expression pattern of fatty acid metabolism-related genes suggests that the epicardial adipose tissue depot appeared to be the primary site of unsaturated fatty acid synthesis in the first week of life in the early neonatal stage, whilst this was dominant in the perirenal depot by day 28 when BAT is, mostly, converted into WAT.

### 7.3.4 Perirenal adipose tissue exhibited a greater tendency to differentiate in response to maternal diet

In order to understand the developmental origins of the molecular changes within fat depots in different anatomical sites in early life, multi-region bioinformatic analyses were conducted on microarray data generated from the three fat depots examined from lambs aged 28 days. The analysis showed that each depot was distinct in its gene expression profile and each contained a small number of unique modules of co-expressed genes, associated with distinct biological processes. The variation between adipose tissue depots in their gene expression profile, differentiation ability and response to local stimulants has been previously reported in mice aged 5–6 weeks (Lee et al., 2017). The perirenal adipose tissue depot was the most responsive to the nutritional intervention compared with the sternal, and epicardial, adipose tissue depots due to its ability to undertake noticeable remodelling with maternal dietary supplementation with canola oil.

### 7.3.5 Maternal canola oil supplementation delays the loss of UCP1 in perirenal adipose tissue

In lamb adipose tissue, the abundance of UCP1 peaked on day 1 and, as expected, decreased with age (Clarke et al., 1997). Maternal fatty acid supplementation with canola oil during lactation resulted in positive stained areas for UCP1 in the perirenal adipose tissue depot on day 28. In contrast, UCP1 was not detected in the perirenal fat of controls or in the sternal depot of lambs in any groups at this age where the tissues were clearly white. Although the specific fatty acids responsible for the effects seen in UCP1 cannot be confirmed due to the many changes that occurred in the milk fatty acid profile, this may be attributable to medium-chain SFAs (Shinohara et al., 2006), which showed a greater decrease in milk in response to the canola oil supplement than with sunflower oil and controls. However, these results are not consistent with UCP1 mRNA expression (qPCR and microarray) and quantification outcomes that showed no effect of dietary supplements on UCP1 gene expression in the three adipose tissue depots. Whilst

the immunohistochemistry analysis will be discussed further in this chapter, variable results between the expression of UCP1 mRNA and protein could be attributed to a weak correlation coefficient between protein and mRNA (Anderson & Seilhamer, 1997) along with the upregulation of IGF2BP2/IMP2 that has a role in inhibiting the translation of mRNAs bearing UCP1 into protein (Dai et al., 2015; Frau et al., 2017). Although IGF2BP2/IMP2 gene expression was not tested in the present study, analysing the microarray data revealed an upregulation of IGF2BP2/IMP2 expression upon maternal supplementation with canola oil (data shown in Appendix 2). More importantly, UCP1 mRNA has been reported to degrade at a faster rate than proteins (Gospodarska et al., 2015).

Interestingly, the lack of change in the expression of brite genes (beige or brown in white) is consistent with the absence of fatty acid supplementation effects on UCP1 expression and suggests that the increase in UCP1 abundance probably resulted from the role of fatty acids in retaining BAT rather than the recruitment of BRITE adipose tissue.

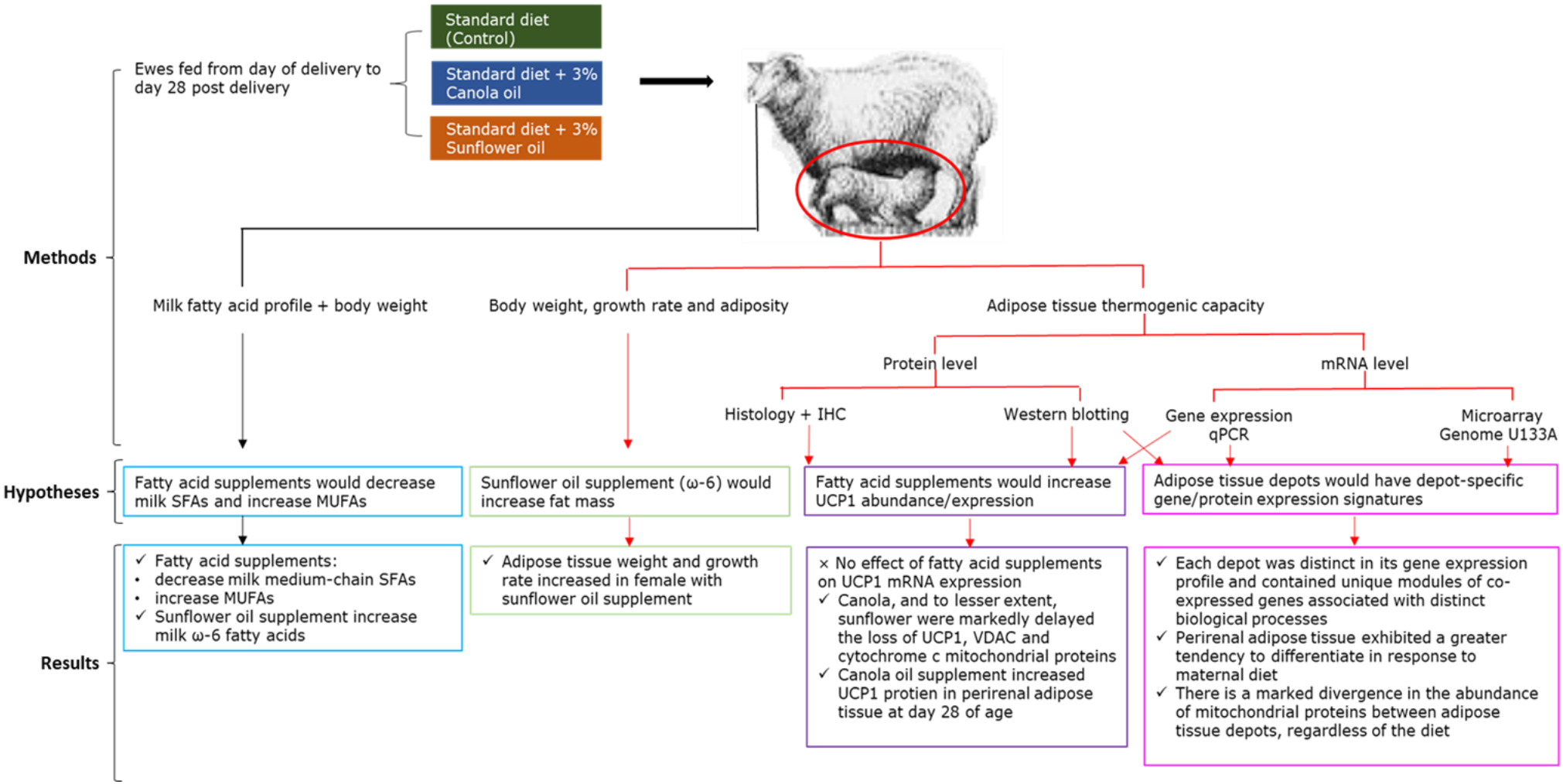



**Figure 7.1 Summary of methods, hypotheses and main results.**

Ewes in the control group were fed a standard diet, ewes in the canola group were fed a standard diet + 3% canola oil and ewes in the sunflower group were fed a standard diet + 3% sunflower oil. Offspring lambs were mother-fed from birth until tissue sampling day. All experiments were performed on day 7 and 28 of age for ewes (black arrows diagram) and lambs (red arrows diagram). Marks indicate whether the hypothesis has been verified (✓) or not (x).

## 7.4 Study limitations

### 7.4.1 Sheep as a model for early life development in humans

Despite sheep being a good model for studying human development as detailed in Chapter 1 (Section 1.6), they have some limitations for nutritional studies. As herbivores and ruminants, they are clearly different from humans in their digestive system, dietary requirements and some aspects of their energy metabolism. Therefore, the results from my thesis cannot be directly applied to humans. For example, the intervention used in this investigation cannot be directly applied to supplementing lactating women's diets with 3% canola/sunflower oil and expecting the same affect seen in ewe's milk. On the other hand, rumen development in newborn lambs is not complete until c.6-8 weeks of age when they begin to eat solid food (Lane et al., 2000). Furthermore, the suckling process initiates by transiting the milk directly from the oesophagus to the abomasum, the final stomach compartment, which means bypassing the rumen (Ørskov, 1972). Hence, as the lambs studied in this investigation were entirely mother fed up to 4 weeks, this limitation is not a major concern when studying nutrition in young lambs.

While the number of offspring in sheep (maximum triplets) is closer to that in humans than the number of mice offspring (large litters), human mothers tend to have single offspring, so this is another limitation in this investigation. In sheep, fetal number has been found to affect the offspring birth weight (Gardner et al., 2007) and later fat mass (Hancocks et al., 2012). However, no significant differences were seen in the mean birth weight among dietary groups (Chapter 4, Section 4.4.1). As detailed in Chapter 2 (Section 2.2.2), if a ewe had triplets, one lamb was immediately removed so each mother only raised two siblings until day 7 (the first sampling point); thus, the multiple fetal number was partly modified after birth. After this point (from day 7 to day 28), each ewe raised only one lamb.

### 7.4.2 Technical limitations

#### 7.4.2.1 Gene expression

The sheep genome sequence has only relatively recently be published, only became fully available in 2014 (Jiang et al., 2014). Sheep are not commonly used as an animal model and the number of genes available for analysis was limited as there is a relative lack of published primer design expertise and relatively few published primer sequences. Therefore, the design of primers and optimisation took longer than expected from studies in small animals. These practical considerations limited potential investigations into omega 6/9 fatty acid pathways (Simopoulos, 2016) in thermogenesis.

Another limitation is that an even distribution of offspring by sex could not be attained and there was unfortunately an imbalance between the number of males and females. As there was no significant difference in the outcomes between males and females within groups in gene expression analyses, the results from both sexes were combined. However, it would have been advantageous to have a greater number of animals in each group to examine potential differences between genders.

Finally, as epicardial adipose tissue is a small depot in a young lamb, there were insufficient remaining tissue samples from epicardial adipose tissue to yield enough mRNA concentration from all animals, so the sample numbers were lower than those for other adipose tissue depots in both time points. However, data analysis shows epicardial tissue was significantly the site of the highest mRNA gene expression in the majority of the genes examined, so this reduction in sample numbers is unlikely to have skewed those results and conclusions.

#### 7.4.2.2 Immunohistochemistry analysis

Although immunohistochemistry is an important auxiliary method for quantifying protein expression (Fritz et al., 1989; Polak et al., 1975), it has limitations in practice and results evaluation. It is common to have weak staining or unwanted background

staining. However, using the semi-automatic machine 'BondMax™ IHC slide processor' and secondary antibodies that act as a detection system was valuable in error reduction (Brigati et al., 1988; Leong, 1993). In addition, (as detailed in Chapter 2, Section 2.3.4.2) histological slices of negative and positive controls were included in each run, and submitted to the same stages of the reaction in order to validate the findings and their interpretation. In evaluating IHC results, the relative percentage of cells positively immunoblotting in relation to the total number of target cells is commonly assessed (De Matos et al., 2010). The main limitation of this method is that it does not show the intensity of IHC staining, indicating a lack of information on slight differences in protein expression between comparative tissues. However, this is helpful in preventing interpretational error resulting from variations in staining intensity between the comparative tissues because of differences in sample status, especially for samples obtained from lambs at 28 days of age, and in processed sample batches.

Although it has been demonstrated that the result from the quantification of the IHC correlates significantly with protein levels assessed through other measurement methods including Western blotting (De Matos et al., 2010), this was not the case in this investigation. It would be beneficial to have more histological slices in each group since some outcomes, in particular perirenal and epicardial in the canola group, showed a trend difference without reaching statistical significance.

#### 7.4.2.3 Protein expression

Unfortunately, Western blotting did not assess UCP1 abundance in the epicardial adipose tissue. This was due to insufficient remaining tissue samples to yield sufficient protein from mitochondrial preparations.

Although it would have been advantageous to confirm gene expression findings on a protein level, antibodies that have been tested to measure protein in sheep are few and not commercially available. Therefore, the majority of gene expression work was conducted using mRNA expression.

### 7.4.3 Variations in fat intake between groups

In both supplemented groups, ewes received an extra 3% of calories from canola or sunflower oil that the control group did not. Therefore, the alteration seen in milk fatty acid profile could be attributed to the increase in fat intake, and thus increased energy intake, as well as the type of supplemented fat. Although ewes in supplemented groups were receiving more kilocalories/day than those in the control group, the percentage of this increase of total dietary intake on kg/kg ewe body weight basis is very small (metabolizable energy= 215 kcal/day). Consequently, because both supplemented groups that received the same percentage of fat supplement revealed variations in their milk fatty acids profiles, it is likely that this is due to the type of fat. Interestingly, the fat content of milk was not affected by the fat supplements, suggesting that the effect of variations in milk fatty acid profile on suckling lambs is attributed to individual milk fatty acid content rather than the total fat content.

## 7.5 Future work

### 7.5.1 Effects beyond 28 days

Whereas the rate of decline in UCP1 within perirenal adipose tissue depots was significantly altered by maternal canola oil supplementation during lactation, it would be useful to investigate this over a longer period, such as until lamb weaning age, which is usually between 12 and 14 weeks; this would allow one to assess whether the supplement prevents the offspring's loss of UCP1 or only delays it. Furthermore, it would be useful to measure the abundance of UCP1 protein after termination of the supplement to explore whether the UCP1 increase is retained permanently. If UCP1 levels revert to that of controls, the supplement would be less important.

### 7.5.2 Direct supplementation with fatty acids

As mentioned before, it is not possible to attribute the effects seen on offspring, particularly UCP1 abundance, after maternal fatty acid supplementation to any single

fatty acid. In order to do this, it would be ideal to supplement ewes/lambs directly with a specific fatty acid such as individual SFAs, LC-PUFA ω-3 or ω-6 to test their role in enhancing or inhibiting UCP1 abundance. Direct supplementation with one of these fatty acids could be a more appropriate strategy to enrich adipose tissues than the indirect supplementation with fatty acid precursors such as canola/sunflower oil. For instance, data showed that the conversion of precursors into PUFA ω-3 fatty acids is limited and inefficient in humans (Anderson & Ma, 2009).

### 7.5.3 Identification and characterisation of microRNAs in the adipose tissues

Integrating miRNA and mRNA expression profiling would be a valuable source to uncover miRNAs underlying thermogenesis in early life (Zhou et al., 2017) and how it would be affected by dietary supplementations. This could be achieved by examining the expression profile and functional characterization of miRNAs throughout sheep adipose tissue development with further focus in gene targets of related miRNAs that may be involved in regulating thermogenesis and fat deposition. Furthermore, further studies can be conducted to establish the correlation between key miRNAs and their target genes in vitro and illustrate the functional impacts that miRNAs serve during adipose tissue development. This would provide novel insights into the development of dietary intervention strategies to increase BAT development.

## 7.6 Final remarks

This study demonstrates how adipose tissue depots differ in their characteristics, gene expression profile and response to changes in the maternal diet. In addition, it has found that manipulating the fatty acid profile of milk ingested by the newborn may delay, or even prevent, UCP1 loss in early life at least in perirenal adipose tissue. The findings in this study could in part help expand the current understanding of adipose tissue development and clarify the variations of its response to dietary intervention,

thus, it could benefit efforts to design and test dietary interventions to prevent/treat obesity.

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# Appendix 1: List of suppliers

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| **ABgene** | Abgene House, Blenheim Road, Epsom, KT19 9AP, UK.<br>www.abgene.com |
| **Affymetrix UK Ltd** | Wycombe Lane, High Wycombe HP10 0HH, UK.<br>www.thermofisher.com |
| **Agilent Technologies** | 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA.<br>www.agilent.com |
| **Alpha Diagnostic International** | 6203 Woodlake Center, San Antonio, TX 78244, USA.<br>www.4adi.com |
| **Alpha Laboratories Ltd** | 40 Parham Dr, Eastleigh, SO50 4NU, UK.<br>www.alphalabs.co.uk |
| **Ambion** | 850 Lincoln Centre Drive, Foster City, CA 94404, USA.<br>www.ambion.com |
| **Anglia Scientific** | 850 Lincoln Centre Drive, Foster City, CA, 94404, USA.<br>www.appliedbiosystems.com |
| **Applied Biosystems** | Lingley House 120 Birchwood Boulevard, Warrington, Cheshire, WA3 7QH, Uk.<br>www.appliedbiosystems.com |
| **BD** | Becton Dickinson and Company, Belliver Industrial Estate, Belliver Way, Plymouth, Devon PL6 7BP, UK.<br>www.bd.com |
| **Bio-Rad Ltd.** | Life Science Research, Education, Process Separations, Food Science, 2000 Alfred Nobel Drive, Hercules, California 94547, USA.<br>www.bio-rad.com |
| **Dako** | Cambridge House, St Thomas Place, Ely, Cambridgeshire, CB7 4EX, UK.<br>www.dako.com |
| **Fisher Scientific UK Ltd** | Bishop Meadow Road, Loughborough, LE11 5RG, UK.<br>www.fishersci.co.uk |

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| **GE Healthcare** | Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.<br><br>www.gehealthcare.co.uk |
| **GraphPad Software** | 7825 Fay Avenue, Suite 230, La Jolla, CA, USA.<br><br>www.graphpad.com |
| **Greiner Bio-One** | Brunel Way, Stroudwater Business Park, Stonehouse, GL10 3SX, UK.<br><br>www.greinerbioone.com |
| **Hamamatsu Photonics (UK) Ltd** | 10 Tewin Rd, Welwyn Garden City, Hertfordshire, AL7 1BW, UK.<br><br>www.hamamatsu.com |
| **Hoefer Inc. MA** | 84 October Hill Rd, Suite 10, Holliston, 01746, USA.<br><br>www.hoeferinc.com |
| **Improvision Ltd** | Coventry, UK<br><br>www.improvision.com |
| **Leica Biosystems** | Balliol Business Park West, Newcastle Upon Tyne, NE12 8EW, UK.<br><br>www.leicabiosystems.com |
| **Leica Microsystems** | Larch House, Woodland Business Park, Breckland, Linford Woods, Milton Keynes, MK14 6FG, UK.<br><br>www.leica-microsystems.com |
| **Manor Farm Feeds** | Green Lane, Owston, Oakham, Leicestershire LE15 8DH, UK.<br><br>www.manorfarmfeeds.co.uk |
| **Media Cybernetics** | 401 N Washington Street, Rockville, 20850, MD, USA.<br><br>www.mediacy.com |
| **Menzel-Gläser** | Glasbearbeitungswerk GmbH & Co., Saarbrückener Str. 248, D-38116, Braunschweig, Germany.<br><br>www.menzel.de |
| **Merial Animal Health Ltd** | Sandringham House, Harlow Business Park, Harlow, Essex, CM19 5QE, UK.<br><br>http://merial.com/ |

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| **Millipore** | Croxley Green Business Park, Watford, Hertfordshire, WD18 8YH, UK.<br><br>www.emdmillipore.com |
| **Nikon UK Ltd** | 380 Richmond Rd, Kingston upon Thames, Surrey, KT2 5DB, UK.<br><br>www.europe-nikon.com/en_GB/ |
| **Objective Imaging Ltd** | Unit 1, Quy Court, Colliers Lane, Stow cum Quy, Cambridge, CB25 9AU, UK.<br><br>http://www.objectiveimaging.com |
| **Partek Inc.** | 624 Trade Center Boulevard, St. Louis, Missouri 63005, USA.<br><br>www.partek.com |
| **Primer Design** | Millbrook Technology Campus, Second Avenue, Southampton, Hampshire, SO15 0DJ, UK.<br><br>www.primerdesign.co.uk |
| **Qiagen** | Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK.<br><br>www.qiagen.com |
| **QImaging, Surrey** | 19535 56th Avenue, Surrey, BC V3S 6K3, Canada.<br>www.qimaging.com |
| **Raytek** | 26 Norton Park View, Sheffield, S8 8GS, UK.<br><br>www.raytek.co.uk |
| **Sakura Finetek** | Flemingweg 10A, 2408 AV Alphen aan den Rijn, Netherlands.<br><br>www.sakura.eu |
| **Sarstedt** | Sarstrabe 1, 51588 Nümbrecht, Germany.<br><br>www.sarstedt.com |
| **Scientific Laboratory Supplies** | Orchard House, The Square, Hessle, East Riding of Yorkshire, HU13 0AE, UK.<br><br>www.scientificlabs.co.uk |
| **Sigma-Aldrich Company** | The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK.<br><br>www.sigma-aldrich.com |
| **Simport Scientific** | 2588 Bernard-Pilon Street, Beloeil, QC J3G 4S5, Canada.<br><br>www.simport.com |

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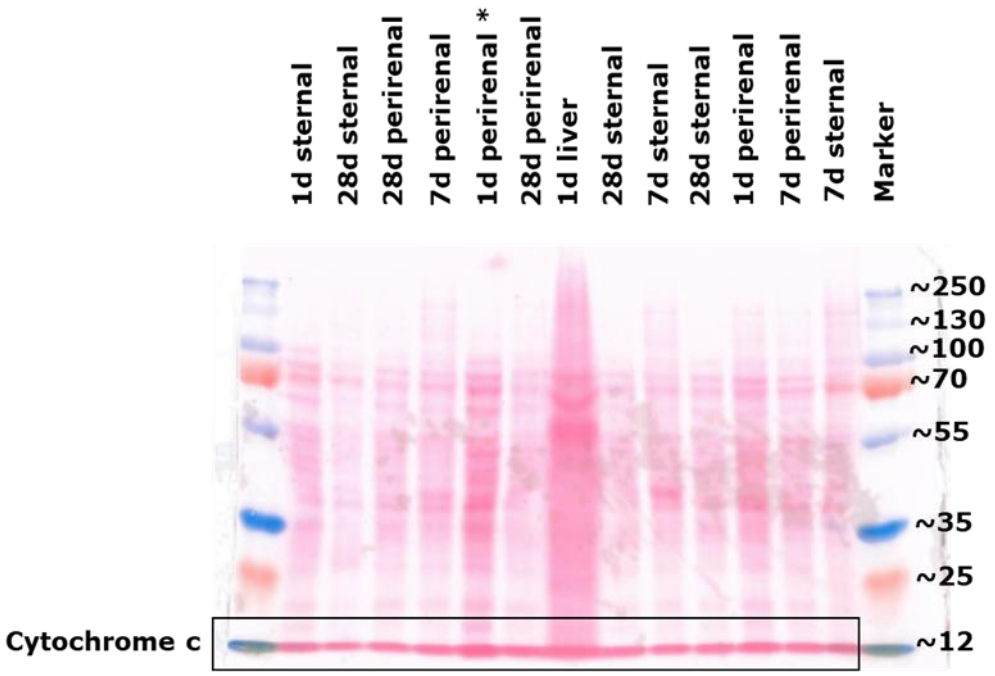
| **Thermo Scientific** | Sarstrabe 1, 51588 Nümbrecht, Germany.<br><br>www.sarstedt.com |
| **Thermo Shandon** | 93-96 Chadwick Road, Runcorn, Cheshire, WA7 1PR, UK.<br><br>www.thermofisher.com |
| **Thistle Scientific** | DFDS House, Goldie Road, Uddingston Glasgow G71 6NZ.<br><br>www.thistlescientific.co.uk |
| **TouchGene Gradient, Techne** | Hinxton Road, Duxford, Cambridgshire, CB2 4PZ, UK.<br><br>www.techne.com |
| **VWR** | Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, UK.<br><br>www.vwr.com |

## Appendix 2: Representative image of Western immunoblot of cytochrome c in lamb's perirenal and sternal adipose tissues from control group.

The numbers in the top indicate lambs age in days. * indicates the reference mitochondrial sample that was loaded on each gel in order to calibrate the results. The molecular mass of cytochrome c is approximately 12 kDa. Ponceau protein stain of the transfer membrane indicating approximately equal loading across the gel. Lane 1d liver shows slightly increased loading which is consistent with the higher level of cytochrome c in the blot compared with adipose tissues.



## Appendix 3: List of genes discussed in co-expressed modules in the perirenal adipose tissue depot.

| Gene Symbol | Gene Name | Average expression | P.Value | Adjusted P.Value |
|---|---|---|---|---|
| NR3C1 | nuclear receptor subfamily 3 group C member 1 | 10.95994 | 1.04E-05 | 0.049671 |
| CYB5B | cytochrome b5 type B (outer mitochondrial membrane) | 2.591708 | 1.82E-05 | 0.049671 |
| GLTSCR1L | GLTSCR1 like | 6.109096 | 1.86E-05 | 0.049671 |
| FAT4 | FAT atypical cadherin 4 | 2.780559 | 1.94E-05 | 0.049671 |
| HOXA5 | homeobox A5 | 7.249847 | 0.000152 | 0.201729 |
| HOXB5 | homeobox B5 | 5.534156 | 0.017121 | 0.65834 |
| HIF1A | hypoxia inducible factor 1 alpha subunit | 9.437446 | 0.030555 | 0.721174 |
| SERP1 | stress-associated endoplasmic reticulum protein 1 | 8.014725 | 0.034117 | 0.735923 |
| KDELR2 | KDEL endoplasmic reticulum protein retention receptor 2 | 6.051192 | 0.041292 | 0.750349 |
| HOXB6 | homeobox B6 | 6.74113 | 0.089144 | 0.798473 |
| DECR1 | 2,4-dienoyl-CoA reductase 1, mitochondrial | 7.694558 | 0.132174 | 0.798473 |
| HOXB8 | homeobox B8 | 4.007776 | 0.194574 | 0.798473 |
| UCP1 | uncoupling protein 1 (mitochondrial, proton carrier) | 2.597627 | 0.375471 | 0.800864 |
| PPARG | peroxisome proliferator activated receptor gamma | 3.390094 | 0.660116 | 0.905129 |
| GHR | growth hormone receptor | 7.425365 | 0.679354 | 0.913187 |

## Appendix 4: List of genes discussed in co-expressed modules in the epicardial adipose tissue depot.

| Gene Symbol | Gene Name | Average expression | P.Value | Adjusted P.Value |
|---|---|---|---|---|
| RQCD1 | RCD1 required for cell differentiation1 homolog (S. pombe) | 3.116614 | 0.68223 | 0.94473 |
| EDF1 | endothelial differentiation-related factor 1 | 2.871342 | 0.95899 | 1 |
| GDF11 | growth differentiation factor 11 | 3.444737 | 0.77087 | 0.961783 |
| ATRAID | all-trans retinoic acid induced differentiation factor | 5.312711 | 0.39275 | 0.896056 |
| CCAR1 | cell division cycle and apoptosis regulator 1 | 6.636831 | 0.02697 | 0.896056 |
| FZR1 | fizzy/cell division cycle 20 related 1 | 4.479714 | 0.2252 | 0.896056 |
| CDC5L | cell division cycle 5 like | 2.735602 | 0.16054 | 0.896056 |

## Appendix 5: List of genes discussed in co-expressed modules in the sternal adipose tissue depot.

| Gene Symbol | Gene Name | Average expression | P.Value | Adjusted P.Value |
|---|---|---|---|---|
| HOXA5 | homeobox A5 | 5.144083 | 0.064078 | 0.809244 |
| HIF1A | hypoxia inducible factor 1 alpha subunit | 9.066826 | 0.031007 | 0.809244 |
| GHR | growth hormone receptor | 6.546327 | 0.978659 | 1 |
| DECR1 | 2,4-dienoyl-CoA reductase 1, mitochondrial | 7.042981 | 0.009683 | 0.809244 |
| PPARG | peroxisome proliferator activated receptor gamma | 2.849863 | 0.101054 | 0.809244 |

## Appendix 6: IGF2BP2 gene expression in examined adipose tissue depots in lambs at day 28 of age in control and canola oil supplemented groups.

| | **Epicardial** | **Perirenal** | **Sternal** |
|---|---|---|---|
| Control | 2.210490361 | 0.743054484 | 1.231966 |
| Canola | 3.151331 | 1.919365 | 1.467435877 |
| P.Value | 8.98E-05 | 2.93E-12 | 9.01E-05 |
| adj.P.Val | 0.003503833 | 1.51E-08 | 0.00350585 |

## Appendix 7: Summary of p values for tow-way AVOVA between controls adipose tissue depots at day 1, 7 and 28 of age.

| **Bonferroni's multiple comparisons test** | **Significant** | **Adjusted P Value** |
|---|---|---|
| Sternal:Day 1 vs. Sternal:Day 7 | ns | 0.1475 |
| Sternal:Day 1 vs. Sternal:Day 28 | **** | <0.0001 |
| Sternal:Day 7 vs. Sternal:Day 28 | *** | 0.0003 |
| Pererinal:Day 1 vs. Pererinal:Day 7 | ns | 0.0811 |
| Pererinal:Day 1 vs. Pererinal:Day 28 | **** | <0.0001 |
| Pererinal:Day 7 vs. Pererinal:Day 28 | ns | 0.5507 |
| Epicardial:Day 1 vs. Epicardial:Day 7 | ** | 0.0029 |
| Epicardial:Day 1 vs. Epicardial:Day 28 | **** | <0.0001 |
| Epicardial:Day 7 vs. Epicardial:Day 28 | ns | 0.2085 |
| Sternal:Day 1 vs. Pererinal:Day 1 | ns | >0.9999 |
| Sternal:Day 1 vs. Epicardial:Day 1 | ns | >0.9999 |
| Pererinal:Day 1 vs. Epicardial:Day 1 | ns | >0.9999 |
| Sternal:Day 7 vs. Pererinal:Day 7 | ns | >0.9999 |
| Sternal:Day 7 vs. Epicardial:Day 7 | ns | >0.9999 |
| Pererinal:Day 7 vs. Epicardial:Day 7 | ns | >0.9999 |
| Sternal:Day 28 vs. Pererinal:Day 28 | ns | >0.9999 |
| Sternal:Day 28 vs. Epicardial:Day 28 | ns | >0.9999 |
| Pererinal:Day 28 vs. Epicardial:Day 28 | ns | >0.9999 |
| **Interaction between adipose tissue depots and age** | **ns** | ***P*=0.4229** |
| **Variability among adipose tissue depots** | **ns** | ***P*=0.2160** |
| **Variability among ages** | **** | **<0.0001** |

ns: not significant.