THE EFFECTS OF VARYING MATERNAL DIETARY FAT QUANTITY AND COMPOSITION ON DISEASE PROGRAMMING IN THE OFFSPRING: A FOCUS ON ESSENTIAL FATTY ACIDS

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FOREWORD

This thesis contains original research in a style accepted for or suitable for publication. As such, results chapters are in the format of a manuscript each containing their own introduction, methods, results and discussion sections. Due to the succinctness of the methods sections prepared for manuscripts, a detailed methods section has also been included as a standalone chapter. Where applicable, manuscripts in their published or accepted format have been included as an appendix.

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PUBLICATIONS ARISING FROM THIS THESIS

Draycott, SAV, Liu, G, Daniel, ZC, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Maternal dietary ratio of linoleic acid to alpha-linolenic acid during pregnancy has sex-specific effects on placental and fetal weights in the rat. *Nutrition & Metabolism*, 16(1), 1.

Draycott, SAV, George, G, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2020). The effect of maternal dietary fat content and omega-6 to omega-3 ratio on offspring growth and hepatic gene expression in the rat. *British Journal of Nutrition*, 1, 30.

Draycott, SAV, Daniel, Z, Khan, R, Muhlhausler, BS, Elmes, MJ & Langley-Evans, SC (2019). Expression of cholesterol packaging and transport genes in human and rat placenta: impact of obesity and a high-fat diet. *J Dev Orig Health Dis*, 1-6.

RELATED PUBLICATIONS

George, G, **Draycott, SAV**, Muir, R, Clifford, B, Elmes, MJ & Langley-Evans, SC (2019). Exposure to maternal obesity during suckling outweighs in utero exposure in programming for post-weaning adiposity and insulin resistance in rats. *Scientific Reports,* 9(1), 10134.

George, G, **Draycott, SAV,** Muir, R, Clifford, B, Elmes, MJ & Langley-Evans, SC (2019). The impact of exposure to cafeteria diet during pregnancy or lactation on offspring growth and adiposity before weaning. *Scientific Reports,* (accepted manuscript).

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACC	Acetyl-coA carboxylase
ALA	Alpha-linolenic acid
BAT	Brown adipose tissue
BMI	Body mass index
C/EBP	CCAAT/enhancer binding protein
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
COX	Cyclooxygenases
CVD	Cardiovascular disease
DBS	Dried blood spot
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
DOHaD	Developmental origins of health and disease
DPA	Docosapentaenoic acid
ELOVL	Elongation of very long chain fatty acids
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FAS/*Fasn*	Fatty acid synthase
FID	Flame ionisation detector
GC	Gas chromatography
GH	Growth hormone
GPDH	Glycerol-3-phosphate dehydrogenase
HDL	High-density lipoprotein
IGF	Insulin-like growth factor
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LOX	Lipoxygenases
LPL/*Lpl*	Lipoprotein lipase
MetS	Metabolic syndrome

MUFA	Monounsaturated fatty acid
NCD	Non-communicable diseases
PAR	Predictive adaptive response
PG	Prostaglandin
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
SREBP-1c/*Srebf1*	Sterol regulatory element-binding protein (variant 1c)
TAG	Triacylglycerol/triglyceride
TX	Thromboxane
UCP1	Uncoupling protein 1
VPR	Volume pressure recording
WAT	White adipose tissue
WHO	World Health Organisation

ABSTRACT

The nutritional environment that an individual is exposed to during development has been shown to affect growth and fat deposition as well as disease risk in later life. In particular, the effects of a maternal diet high in fat are relatively well characterised in animal models and are typically associated with a range of offspring phenotypes, including high blood pressure, increased adiposity and impaired insulin and glucose homeostasis. These high-fat diets often contain high amounts of saturated fatty acids (SFA), however, changes in dietary recommendations over the past 60 years have resulted in increased consumption of vegetable oils. As such, dramatic increases in the intake of omega-6 polyunsaturated fatty acids (PUFA), specifically linoleic acid (LA), have been observed in Western populations. This increase in omega-6 fatty acid consumption has not been matched by an increase in the intakes of omega-3 fatty acids, which has remained stable over this period of dietary change. The effect of this dietary pattern on offspring risk of obesity and cardiometabolic disturbance is yet to be established, however, studies have demonstrated a pro-adipogenic effect of omega-6 fatty acids suggesting increases in the intake of LA may be linked to the increasing prevalence of obesity.

Broadly, this research aimed to elucidate the effects of a maternal dietary LA to alpha-linolenic acid (ALA; omega-3) ratio similar to that consumed in the Western diet (9:1) compared to a proposed 'ideal' ratio of 1:1.5, on offspring growth, fat deposition and potential markers of cardiometabolic disturbance. Due to the increasing amount of women of reproductive age consuming a high-fat diet and, since total dietary PUFA intake also influences PUFA metabolism, we also investigated the effect of feeding each dietary fat ratio at either 18% fat w/w (in line with dietary recommendations) or at a higher fat level of 36% fat w/w. A rat model was utilised to achieve the study objectives by allowing for tight control of dietary manipulation as well as invasive end points.

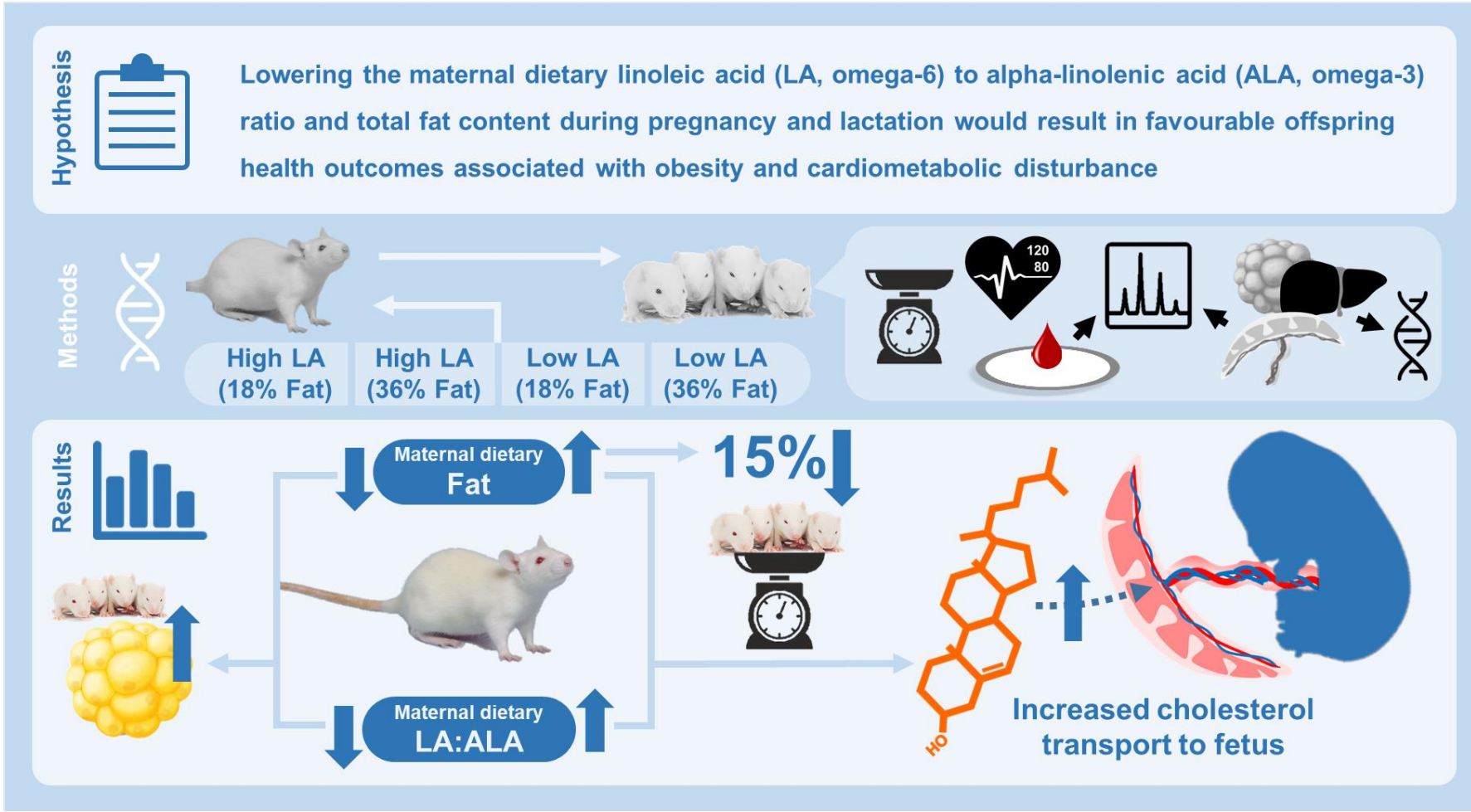
The main findings of this research have demonstrated that a low LA:ALA ratio in the diet was effective at increasing circulating levels of long chain omega-3 PUFA, specifically eicosapentaenoic acid (EPA), in the dams which was largely reflected in the offspring. However, improving the omega-3 status of the offspring had no discernable beneficial effect on adiposity during early-life. In fact, a maternal low LA,

low fat diet was associated with increased fat mass in the offspring. Whether the fluctuations in the lipogenic capacity of tissues observed in these studies in the offspring during early-life is indicative of later metabolic disturbance remains to be established. Further to this, a maternal high fat diet, irrespective of the LA:ALA ratio, was associated with a 15% reduction in bodyweight in both male and female offspring when compared to a low fat diet ($P<0.0001$). This was independent of any maternal weight gain and was persistent throughout early-life.

Finally, maternal consumption of a high LA, high fat diet was associated with significant upregulation of genes associated with cholesterol packaging and transport across the placenta. This implicates the placenta as a potential mechanistic mediator of the effects of maternal diet on offspring health. This hypothesis was also tested in pregnant women of different body mass index (BMI) classifications. In women, however, maternal BMI was associated with fewer inconsistent alterations in gene expression. However, the absence of nutritional intake data from these participants was a limitation of the study and comparability to rodent data.

In summary, the results of this thesis indicate that the adipogenic effects previously associated with omega-6 fatty acids do not appear to be contributing towards increased risk of adiposity in offspring when consumed in the maternal diet. The capacity for early-life alterations in tissue function and structure to elicit long-term consequences for individuals remains to be established and should be a focus of future studies. However, a maternal high fat diet, where the predominant fat source was not saturated fats, still elicited unfavourable outcomes in the offspring despite no evidence of increased weight gain or adiposity in the mothers. This has the potential to impact on recommendations to expectant mothers around reducing the quantity of fat within their diet even if it has not had any unfavourable phenotypic effects on the individuals themselves. Future studies should consider more mechanistic approaches however, the preliminary investigations in this thesis highlight the placenta as a likely contributor to the observations between maternal diet and offspring health.

VISUAL ABSTRACT



1 INTRODUCTION

1.1 Obesity

1.1.1 Definitions and classifications

Overweight and obesity are defined by the World Health Organisation (WHO, 2017a) as "abnormal or excessive fat accumulation that presents a risk to health". Measurement of a person's body mass index (BMI), a formulation of weight divided by height squared (kg/m^2), remains the most common indicator of obesity in adults. Current classifications state that an adult with a BMI 25-29.9 kg/m^2 is considered overweight and obese if their BMI $\geq$ 30 kg/m^2 (WHO, 2017a). The use of BMI as an inexpensive population measure is central to its widespread use, however, it does possess limitations as a direct proxy for the estimation of obesity. BMI does not take into account factors such as sex, age, ethnicity, all of which affect the balance of muscle and fat in the body, or fat distribution. A study by Romero-Corral *et al.* (2008), for example, showed that BMI underestimated obesity such that it failed to identify 50% of participants who were defined as obese through body fat percentage measurements. The same study reported that BMI failed to discriminate between body fat percentage and lean mass particularly in men and elderly participants, a common criticism of the method (Frankenfield *et al.*, 2001). As such, waist circumference and body fat percentage are often used in conjunction with BMI for a more accurate assessment of overweight or obesity in an individual (Burton, 2010). Due to the confounding effect of developmental age on the relationship between weight and the distribution of fat and muscle, and thus the accuracy of BMI measurements, they are not used for the classification of overweight and obesity in children. Instead, reference is made to population based measurements specific for age and sex and classification is based on how many standard deviations an individual's weight falls from the population mean (WHO, 2017a).

1.1.2 Prevalence of obesity and future projections

Despite disagreements in the measurement criteria for the diagnosis of obesity, BMI continues to be the gold-standard for adult diagnosis and the WHO estimated a staggering 1.9 billion adults to be overweight or obese as of 2016 (WHO, 2017a). This

largely preventable condition, previously considered an issue for high-income countries, is now on the rise in low- and middle-income countries and is not only affecting adults. Of increasing concern is that 41 million children under the age of 5 were estimated to be overweight or obese in 2016 (WHO, 2017a). Furthermore, the prevalence of obesity worldwide continues to rise (Finucane *et al.*, 2011) and, beyond the individual health risks, places a huge social and economic burden on afflicted countries.

Based on current trends, modelling estimates predict that over half of the UK population could be overweight or obese by 2030, leading to an economic burden of around £1.9-2 billion per year in healthcare costs associated with overweight and obesity as well as its co-morbidities (Wang *et al.*, 2011). More recent modelling approaches have used available data from longitudinal studies in the US. Using these data the researchers were able to simulate growth trajectories of children into adulthood and predicted that, based on representative anthropometrics of the population, the majority of children within the population (57.3%; based on 2016 data) would be obese at the age of 35 years (Ward *et al.*, 2017). Further to this, the same study suggested that for roughly half of these individuals, the onset of obesity would occur during childhood. Another study in the Australian population predicted that, whilst the prevalence of overweight was likely to remain relatively steady until the year 2025, the prevalence of obesity and severe obesity was predicted to rise to 35% and 13% respectively during this time (Hayes *et al.*, 2017). This equates to a respective increase of 8% and 4% based on 2014-2015 data (Australian Institute of Health and Welfare, 2017).

1.1.3 Causes: a nutritional perspective

The primary cause of obesity is an "energy imbalance between calories consumed and calories expended" (WHO, 2017a). A positive energy balance is where energy intake is greater than energy expenditure leading to increased energy storage within the body. Prolonged exposure to a positive energy balance leads to weight gain and ultimately to obesity. Whilst seemingly quite a simplistic explanation, it is important to note that obesity is not merely a result of overindulgence and lack of physical activity. There are many factors affecting an individual's susceptibility to weight gain and obesity, both biological (genetics, sex, age, and ethnicity) and environmental (e.g.

socioeconomic status, stress), that should not be overlooked. Further to this, there appears to be evidence to suggest that regulation of body weight is asymmetric (Müller *et al.*, 2010). Likely due to the more immediate threat to survival that undernutrition possesses, stronger feedback mechanisms exist to restore normal energy balance in states of undernutrition as opposed to overnutrition (Blundell and King, 1996).

Despite the complexity of obesity aetiology, prevention and treatment, nutritional status and physical activity remain at the core of this issue and it is often through the interference of nutritional status that other external factors associated with obesity act. For example, many of the genes associated with a heritable predisposition to obesity centre around hunger, satiety and food intake (O'Rahilly and Farooqi, 2008). Similarly, the association between low socioeconomic status and increased incidence of obesity appears to be due to unhealthier food choices driven by the cost of food items (Pechey and Monsivais, 2016).

Whilst all macronutrients are essential to the diet and should not be excluded, excess dietary fat has commonly been associated with weight gain and obesity (Bray *et al.*, 2002). Dietary fat has a higher energy density than protein and carbohydrate and, whilst the storage of protein and carbohydrate is relatively limited, the capacity for fat storage is virtually unlimited. Global increases in the incidence of overweight and obesity can be attributed to many factors, although global shifts in dietary habits tending towards an increased energy intake coupled with a decrease in physical activity are often implicated as a primary cause.

1.1.4 Health consequences of overweight and obesity

The health consequences associated with obesity are wide reaching, ranging from increased risk of premature death to several non-fatal debilitating diseases and psychological issues (Table 1.1). Whilst obesity is often considered a disease in its own right, overweight and obesity are strongly associated with an increased risk of type 2 diabetes (Resnick *et al.*, 2000, Janssen, 2007), various cancers (Bianchini *et al.*, 2002) and the leading causes of death worldwide, coronary heart disease (CHD) and stroke (Kenchaiah *et al.*, 2002, WHO, 2017b). Obese individuals often present with one or more co-morbidities including hypertension, glucose intolerance, dyslipidaemia, hypertriglyceridemia and decreased high-density lipoprotein (HDL)

cholesterol. The presence of at least three of these conditions in an individual is referred to as the Metabolic Syndrome (MetS), and is associated with an increased risk of type 2 diabetes (Galassi *et al.*, 2006) and a 2-fold higher risk of a cardiovascular event (Mottillo *et al.*, 2010).

Several robust studies have reported a clear relationship between excessive body weight and increased mortality (Adams *et al.*, 2006, Berrington de Gonzalez *et al.*, 2010, Calle *et al.*, 1999, Chen *et al.*, 2013, Whitlock *et al.*, 2009). A comprehensive study by the Global Burden of Disease 2015 Obesity Collaborators systematically investigated this association in 195 countries worldwide over a 25-year period (Afshin *et al.*, 2017). The authors of this study reported that over 4 million deaths worldwide were accounted for by overweight and obesity in 2015, 70% of which were due to cardiovascular disease (CVD).

Many of the co-morbidities associated with obesity in adults are now being observed more commonly in children (Table 1.1). Further to this, it is estimated that 80% of obese adolescents will continue to be obese in adulthood resulting in an increased risk of the plethora of issues associated with adult obesity (Simmonds *et al.*, 2016). Obesity in adolescence has been directly linked to increased risk of death from CHD and atherosclerosis, and this effect is independent of adult weight (Must *et al.*, 1992). However, more recent systematic reviews have highlighted that there is little evidence to support childhood obesity as an independent risk factor for CVD (Lloyd *et al.*, 2010) and MetS (Lloyd *et al.*, 2012) and, in fact, more adverse outcomes are observed when individuals are of a normal, or even low, BMI during childhood and become obese in adulthood. This highlights the complex relationship between early BMI and adult disease risk and that targeting health management practices at overweight and obese children only may result in the neglect of a high-risk group of individuals. Adult obesity, however, is a major independent risk factor for an abundance of diseases and childhood obesity is a major risk factor for adult obesity. As such, whilst it is important to maintain interventions targeting weight loss and management in adults in order to prevent the neglect of these high-risk groups, prevention in early life may be critical to reducing this obesity epidemic.

Table 1.1 Health consequences associated with obesity in children and adults (Adapted from WHO (2000)).

	Children	Adults
High prevalence	• Faster growth • Psychosocial problems • Persistence into adulthood (for late onset and severe obesity) • Dyslipidaemia	• Type 2 diabetes • Gallbladder disease • Dyslipidaemia • Insulin resistance • Breathlessness • Sleep apnoea
Intermediate prevalence	• Hepatic Steatosis • Abnormal glucose metabolism • Persistence into adulthood (depending on age of onset and severity)	• CHD • Hypertension • Osteoarthritis (knees) • Hyperuricemia and gout
Low prevalence	• Orthopaedic complications • Sleep apnoea • Polycystic ovary syndrome • Pseudotumor cerebri • Cholelithaiasis • Hypertension	• Cancer (breast, endometrial and colon) • Reproductive hormone abnormalities • Polycystic ovary syndrome • Impaired fertility • Lower back pain • Increased risk of anaesthesia complications • Fetal defects associated with maternal obesity

1.2 Developmental origins of health and disease (DOHaD)

1.2.1 An introduction to the DOHaD hypothesis

Fetal development has previously been considered to be under precise genetic control, however, it is now apparent that plasticity of the cells during the developmental period presents a window of susceptibility in which, when subject to insult, the offspring can undergo physiological changes which can have a lifelong impact (Bateson *et al.*, 2004). This phenomenon has been termed ‘programming’ (Lucas, 1991, Lucas, 1994). Suggestions have been made that the physiological adaptations of the fetus in response

to changes in the intrauterine environment, such as alterations in the quality and quantity of maternal nutrients, may result in changes in gene expression which can in turn impact upon tissue structure. Owing to the fact that tissue structures are generally in place at the time of birth, or shortly after, the impact of a relatively short period of insult can potentially become permanent. This can in turn lead to a predisposition to diseases in later life, and this concept forms the basis of the Developmental Origins of Health and Disease (DOHaD) hypothesis, pioneered by the work of David Barker and colleagues (Barker *et al.*, 1990, Hales *et al.*, 1991).

1.2.1.1 Evidence from epidemiological studies

Support for the DOHaD hypothesis was initially provided by epidemiological studies investigating associations between early life factors and a range of diseases in later life. Barker *et al.* (1989) found that in a group of men from Hertfordshire, all of whom were born between 1911 and 1930, those with the lowest weights at birth and at one year of age had the highest death rates from ischaemic heart disease. Further studies in this population, as well as others, provided evidence of inverse associations between birthweight and risk of hypertension (Barker *et al.*, 1990), impaired glucose tolerance (Hales *et al.*, 1991) and type 2 diabetes (Barker *et al.*, 1993). These findings were supported by a series of studies on a group of men and women in Finland that also found associations between low birthweight and increased risk of death from CHD (Forsén *et al.*, 1997, Forsen *et al.*, 1999). In addition, the Finnish studies also demonstrated that the highest risk of CHD was observed in those individuals who were lighter at birth but had a greater body mass at 7 years of age (Eriksson *et al.*, 1999). As such, the theory emerged that a period of rapid 'catch-up' growth in early life increased an individual's risk of adverse outcomes.

The emergence of further studies demonstrating this same association subsequently led to 'The Thrifty Phenotype' hypothesis. Introduced by Hales and Barker (1992), this hypothesis proposed that poor nutrition in early life was the driver for the epidemiological association between poor fetal and infant growth and subsequent development of type 2 diabetes. Essentially, poor nutrient supply from the mother leads to adaptations within the fetus which protect the individual from the effects of a low nutrient postnatal environment (Figure 1.1). It is naïve to think of this reduction in birthweight as causal in the disease pathway as birthweight itself is a result of a

combination of environmental and genetic factors during fetal life. Whilst reductions in intrauterine growth can be attributed to many factors, including maternal stress (Rice *et al.*, 2010), smoking (Ward *et al.*, 2007) and air pollution (Pedersen *et al.*, 2013), this hypothesis placed inadequate maternal nutrition as a key contributor.

The long-term effects of maternal malnutrition in humans are difficult to study experimentally, however, a number of historical cohorts have provided important 'natural' experiments to demonstrate this phenomena. One of the most studied populations is the Dutch Hunger Winter cohort. During the winter of 1944-1945, western Holland was subject to severe food shortages for approximately 6 months, at the height of which daily intake was reduced to just 400-800 calories. This 'Dutch Hunger Winter' led to a cohort of offspring that had been exposed to severe maternal undernutrition at different times in gestation. Retrospective studies of this cohort have shown that men exposed to the famine in early gestation were more likely to become obese in adulthood, whereas men exposed to the famine in late gestation were less likely to become obese (Ravelli *et al.*, 1976). Further studies of this cohort have demonstrated that exposure to the famine in early gestation was also associated with an atherogenic lipid profile (Roseboom *et al.*, 2000). Several other famine cohorts have been investigated and produced similar associations to those observed in the Dutch Hunger Winter cohort (Hoffman *et al.*, 2017). Taken together, these studies have triggered a cascade of research investigating maternal dietary intake as a central driver of the DOHaD hypothesis.

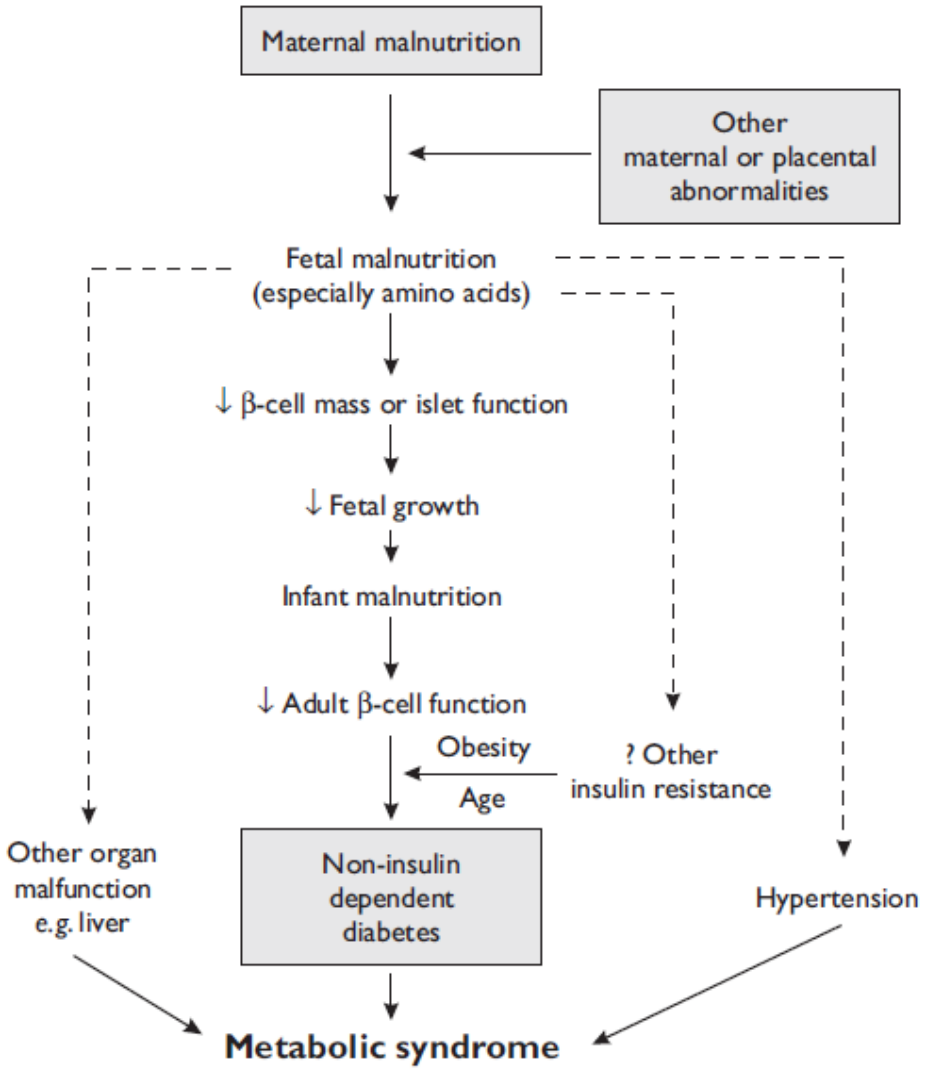


Figure 1.1 Diagrammatic representation of the thrifty phenotype hypothesis (Taken from Hales and Barker (2001))

1.2.1.2 Criticisms of epidemiological studies

The basis on which the DOHaD hypothesis was formed is also the main source of criticism directed towards it. The inability of epidemiological studies to properly account for confounding factors and potential reporting bias are among its major criticisms (Huxley *et al.*, 2002). Further to this, whilst the association between birthweight and later disease risk is compelling, it is just that, an association. The lack of evidence linking maternal nutrition to anthropometric measurements at birth in human cohorts is also a major concern and has led to uncertainty as to whether maternal nutrition really is a key component of this hypothesis. Whilst the Dutch Hunger Winter cohort suggested that maternal undernutrition can influence

birthweight, the dietary insult in these studies was severe whereas changes in birthweight were not (Roseboom *et al.*, 2001), raising questions as to how significant an influence maternal nutrition actually has on fetal growth. Studies of well-nourished populations have also shown that while changes in maternal dietary intakes are associated with differences in birthweight, these differences are small and do not shift birthweight outside the normal range (Englund-Ögge *et al.*, 2018). It is important to note, however, that the nature of DOHaD effects are inherently small, as large perturbations in growth and metabolism are often lethal to the developing fetus. As such small variations within the normal range may still be physiologically important. Nonetheless, overcoming these criticisms required establishment of a direct link between maternal nutrition and anthropometric birth measurements or to disease risk in later life.

1.2.1.3 Evidence from animal models

Whilst epidemiological studies have highlighted a link between early life nutrition and adverse health outcomes in humans, establishing causality is challenging as experimental manipulation of the maternal diet during pregnancy is difficult and often not ethically viable. As a result, animal models have been critical to our understanding of the DOHaD hypothesis and provide significant advantages over epidemiological studies. A range of animal models have been utilised, however, rodents are most commonly used owing to their shorter lifespan and length of gestation, larger litter size, the ability to test more specific hypotheses, and an increased degree of nutritional and environmental control as well as the measurements of invasive end points (McMullen and Mostyn, 2009b). To provide comparability with epidemiological studies and in an attempt to establish a link between maternal nutrition and birthweight, early studies investigated the effects of global nutrient restriction (Woodall *et al.*, 1996). These dietary interventions resulted in reduced offspring birthweight and were associated with hypertension in later life, a key risk factor for CVD (Woodall *et al.*, 1996, Vickers *et al.*, 2000). Studies investigating the impact of key nutrient deficiencies, including protein (Langley and Jackson, 1994, Jackson *et al.*, 2002) and iron (Gambling *et al.*, 2003), have also reported reductions in birthweights and increased blood pressure in the offspring in later life.

The additional evidence from animal studies led to the expansion of the thrifty phenotype hypothesis. The predictive adaptive response (PAR) hypothesis, proposed by Gluckman and Hanson (2004), suggested that an individual can make physiological adaptations to environmental cues at a time of developmental plasticity. Plasticity is highest during early development and so the hypothesis proposes that the fetus can make physiological changes *in utero* to adapt to its predicted postnatal environment. Whilst this level of adaptation could be considered an evolutionary advantage, the hypothesis proposes that if the actual environment is different to that predicted by maternal cues the physiological adaptations of the offspring place them at an increased risk of disease (Figure 1.2).

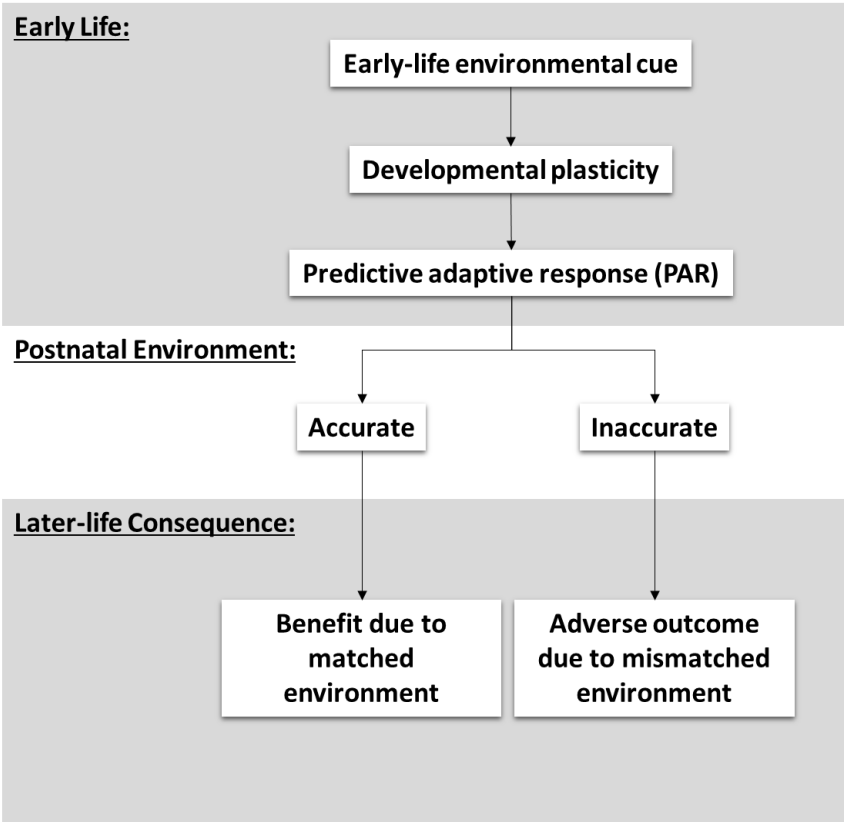


Figure 1.2 Summary of the predictive adaptive response hypothesis. Environmental cues, such as maternal undernutrition, stimulate responses within the developing offspring to ensure it is best suited for the postnatal environment. If the postnatal environment matches the prenatal prediction then the individual is well adapted and is at no greater disease risk. If there is a mismatch in the predicted and actual postnatal environment then disease risk is increased in later life (Adapted from Low *et al.* (2012)).

1.2.1.4 Criticisms of animal studies

Animal studies have provided a much more detailed insight into the associations between specific dietary alterations within the maternal diet on offspring health outcomes. The different species of animal models used, dietary interventions applied and study designs have been extremely diverse (Dickinson *et al.*, 2016). Whilst this is often a criticism of animal studies as it makes comparisons between studies difficult, and combining data in meta-analyses virtually impossible (Ainge *et al.*, 2011, Armitage *et al.*, 2004), it is worth noting that despite these variations the offspring outcomes are relatively conserved and appear to converge on a phenotype associated with the metabolic syndrome (Armitage *et al.*, 2004). This alludes to the possibility of

a few key mechanisms being responsible for the association between the early life nutritional environment and offspring health outcomes (McMullen *et al.*, 2012). The biggest criticism surrounding animal studies is that, like epidemiological studies, they have failed to elucidate an underlying mechanism.

1.2.1.5 Potential mechanisms

Identification of the mechanisms by which nutritional factors at key developmental stages exert long-term or permanent effects is critical to the development of disease prevention strategies. The use of animal models to investigate the DOHaD hypothesis have led to the formation of proposed mechanisms responsible for the phenomenon. Such mechanistic concepts have been reviewed in great detail elsewhere (Langley-Evans, 2013, Langley-Evans, 2009, Langley-Evans and McMullen, 2010, McMullen and Mostyn, 2009a), therefore, the three most documented hypotheses will be briefly considered here.

(1) *Alterations in tissue structure:* It has been postulated that exposure to specific nutrients during fetal and early postnatal life, i.e. critical stages for organ and tissue development, leads to alterations in the proliferation and differentiation steps involved in organ and tissue development. This could lead to transient or permanent alterations in structure and function and several models have demonstrated a reduction in cell number or changes to the composition of cell types in response to a range of maternal dietary alterations (Langley-Evans *et al.*, 1999, Remacle *et al.*, 2007, Camm *et al.*, 2011, Woods *et al.*, 2004).

(2) Exposure to maternal glucocorticoids: Glucocorticoids are important mediators of stress responses, however, under normal conditions their transport across the placenta is closely regulated by 11β-hydroxysteroid dehydrogenase 2 (11βHSD2) which converts active glucocorticoids to inactive ones to prevent excessive accumulation. Fetal exposure to excessive glucocorticoids is associated with growth retardation, hypertension and impaired glucose homeostasis (Dodic *et al.*, 1998, Nyirenda *et al.*, 1998). Further to this, dietary insults have been associated with a reduction in 11βHSD2 expression in the placenta (Langley-Evans *et al.*, 1996b, McMullen *et al.*, 2004). It has therefore been postulated that dietary stress leads to an increase in glucocorticoid transfer to the fetus, through diminished 11βHSD2 capacity, resulting in overexposure

of fetal tissues. This can in turn lead to changes in the expression of the many genes that contain glucocorticoid response elements and hence lead to the alterations in tissue structure and function discussed above.

(3) *Epigenetic modifications:* The most recent mechanism that has been suggested to play a role in the programming of disease are epigenetic modifications. These modifications, which include DNA methylation and histone modifications, silence or switch on genes by altering DNA structure and hence access of proteins required to initiate transcription. In the Dutch Hunger Winter cohort, follow up experiments have identified reduced DNA methylation of the insulin-like growth factor 2 (IGF-2) gene in individuals who were exposed to maternal nutrient restriction (Heijmans *et al.*, 2008). Importantly, these modifications were detected in these individuals 60 years after the nutritional insult, suggesting that the effects are permanent, and providing an attractive putative mechanism for how early-life factors influence disease risk in later-life. Further to this, animal studies have shown that maternal nutrient restriction, overnutrition, obesity and diabetes are all capable of affecting the offspring epigenome (Bianco-Miotto *et al.*, 2017). However, causal relationships between epigenetic modifications, gene expression and development have proven difficult to establish (Altobelli *et al.*, 2013).

1.3 Developmental origins of obesity

The association between maternal diet and offspring disease risk is now considered by many to be well supported by evidence from epidemiological and animal studies. Maternal undernutrition was the focus of many of the studies that established this association. More recently however, undoubtedly due to the increased incidence of obesity in Western populations, the effects of maternal overnutrition and obesity on offspring health are being increasingly subjected to intense study.

A wide range of robust epidemiological studies have shown that a high maternal BMI is associated with an increased offspring BMI and increased adiposity well into adult life (Kaar *et al.*, 2014, Reynolds *et al.*, 2010, Schoppa *et al.*, 2019). There is also evidence that obese women (BMI>30 kg/m^2) are 2-3 times more likely to give birth to a large for gestational age baby (Ehrenberg *et al.*, 2004). Recently, two large scale meta-analyses have confirmed this strong association with one identifying a 264%

increased risk of childhood obesity if the mother was obese before conception (Heslehurst *et al.*, 2019, Voerman *et al.*, 2019). It has been postulated that the positive relationship between maternal BMI and offspring adiposity may, at least in part, be a consequence of increased maternal nutrient intake. Thus, the developing fetus and infant of the obese mother is exposed to an increased nutrient supply via the placenta or through changes in breast milk composition, and this promotes overgrowth and increased adiposity.

In animal models, diets high in saturated fats or high-fat high-sugar 'cafeteria' diets are commonly used to model maternal obesity and overnutrition. These studies have reported that providing animals with these diets during pregnancy and lactation is associated with increased blood pressure (Guberman *et al.*, 2013, Samuelsson *et al.*, 2008), impaired insulin and glucose homeostasis (Taylor *et al.*, 2005, Samuelsson *et al.*, 2008, Akyol *et al.*, 2012) and increased adiposity (Guo and Jen, 1995, Khan *et al.*, 2004, Bayol *et al.*, 2005) in the adult offspring. These observations were supported by Menting *et al.* (2019) who systematically reviewed 145 studies investigating the effect of maternal obesity on offspring cardio-metabolic health. Maternal obesity was associated with increased adiposity, blood pressure, triglycerides (TAG), cholesterol, glucose and insulin levels in the offspring. Interestingly, and despite these associated deleterious outcomes, many of these studies reported no effect of these dietary exposures on birthweight.

Whilst these studies provide insight into possible predictors of obesity risk in later life, they do not always adequately isolate the effects of maternal obesity from other comorbidities, or indeed, isolate the effects of maternal obesity from the impacts of maternal nutrition. Further to this, the limitations associated with epidemiological research do not always allow for examination of key metabolic disturbances in tissues making identification of possible mechanisms difficult.

1.3.1 Placental influence on nutrient transfer

The impact of increased nutrition on the developing fetus and role of the placenta is clearly demonstrated by studies of infants of mothers with pre-gestational or gestational diabetes. In these studies, excess glucose transfer to the developing fetus, as a result of maternal hyperglycaemia, is associated with increased birthweight and

increased offspring adiposity (Plagemann *et al.*, 1997, Kamana *et al.*, 2015). The increased circulating glucose levels in the mother are transferred to the fetus via the placenta resulting in increased fetal glucose concentrations. Maternal insulin does not cross the placenta, however, so the fetal pancreas is responsible for secreting insulin to manage this hyperglycaemia. This results in fetal hyperinsulinemia which has been shown to be associated with both excessive fetal growth (Fowden, 1995, Fowden *et al.*, 1989) and increased adiposity in later life (Silverman *et al.*, 1993). Importantly, it appears that this fetal response is facilitated by physiological changes to the pancreas (such as altered β-cell number) during times of cellular plasticity (Zhang *et al.*, 2011). The failure of the pancreas to return to normal physiology and function postnatally may be one of the major factors responsible for the long-term metabolic issues experienced by individuals following exposure to maternal hyperglycaemia.

Due to the establishment of the processes surrounding maternal hyperglycaemia, a large volume of the literature focuses on obese pregnancies complicated by gestational diabetes. Interestingly, however, a study by Kabali and Werler (2007) demonstrated that non-diabetic obese mothers were also more likely to have babies that were large for gestational age and obese pregnancies have also been associated with alterations in placental structure and function. A study by Brouwers *et al.* (2018) on women with non-complicated pregnancies in the Netherlands found that increased maternal pre-pregnancy BMI was associated with increased placental weight and high-grade chronic villitis which is often associated with alterations in fetal growth. Further to this, studies have shown associations between altered maternal nutrition and placental transfer of fatty acids (Brass *et al.*, 2013), cholesterol (Daniel *et al.*, 2016) and amino acids (Farley *et al.*, 2009).

1.4 Adipose tissue development, maintenance and function

1.4.1 Types of adipose tissue, function and morphology

Adipose tissue, now considered an organ in its own right, is primarily made up of adipocytes but also contains various other cells including blood cells, endothelial cells, immune cells, fibroblasts and pre-adipocytes (Ailhaud *et al.*, 1992). In mammals, there are two distinct types of adipose tissue, brown adipose tissue (BAT) and white adipose tissue (WAT), which differ in their function, morphology, and development.

Exclusive to mammals, BAT is responsible for non-shivering thermogenesis and contributes to maintenance of normal body temperature. Early studies in humans identified that BAT was present in highest quantities during fetal and early life but declined into adulthood (Heaton, 1972) where it is mainly confined to subcutaneous regions (Sacks and Symonds, 2013). WAT is by far the most abundant adipose tissue sub-type in humans and its major role is energy storage in the form of TAG. WAT stores are utilised, and hence deplete, in times when energy expenditure exceeds intake and increase under reverse conditions. More recently it has been demonstrated that WAT has other important physiological roles in addition to energy storage. Thus, WAT has been recognised as an organ capable of major endocrine and secretory functions and has been demonstrated to play a central role in the regulation of inflammatory processes and glucose homeostasis (Trayhurn, 2005, Trayhurn and Beattie, 2001). In contrast to BAT adipocytes, which are around 10-25μm in diameter and are considered multilocular (i.e. containing many fat vacuoles), WAT adipocytes are larger (20-100μm in diameter) and contain one vacuole where TAG are stored as a long-term fuel reserve (Stock and Cinti, 2003). BAT is highly vascularised and contain many mitochondria. These mitochondria expresses a unique protein, uncoupling protein 1 (UCP1), which actively transports protons into the mitochondrial matrix. This process is not 'coupled' with synthesis of energy in the form of ATP and instead produces heat as a bi-product (Cinti *et al.*, 1997). These highly specialised adaptations are conducive to its main function of heat production.

BAT has, in recent years, been investigated as therapeutic target for obesity. This is primarily due to early studies in rodents that showed altered BAT activity in response to obesity (Trayhurn, 2018). However, it is worth noting that rodent BAT development is quite different to that observed in humans. Rodents possess considerable deposits of BAT throughout their adult life due to its key role in maintaining body temperature during times of hibernation where animals are exposed to prolonged periods of cold (Cannon and Nedergaard, 2004). As such, human BAT may play a much less significant role in obesity prevention in humans. For this reason, alongside the function of WAT as a site of unlimited lipid storage and the observation of significant increases in WAT stores in obese individuals, from here the focus will be on WAT rather than BAT.

1.4.2 White adipose tissue (WAT) development

Adipocytes have been detected before birth in many species including humans, however, in rodents they are not detected until after birth (Ailhaud *et al.*, 1992). The development of white adipocytes begins during the second trimester of embryonic development in humans where small adipocyte cell clusters begin to develop (Poissonnet *et al.*, 1984). These clusters aggregate in distinct locations and increase in size as development progresses but the number of clusters remains the same (Ailhaud *et al.*, 1992). Adipocytes are of mesodermal origin and embryonic stem cells undergo various differentiation steps resulting in cells becoming increasingly committed to the adipocyte lineage. Cells committed to the adipocyte lineage are termed pre-adipocytes. Differentiation to mature adipocytes (adipogenesis) occurs in several stages and is regulated by a plethora of transcription factors and signals.

1.4.2.1 Transcriptional regulation of adipogenesis

WAT adipogenesis can occur at any stage during development and adulthood. Studies have shown that cell cycle arrest of pre-adipocytes appears to be a requirement for differentiation (Pairault and Green, 1979). Early markers of the commitment of cells to the adipocyte lineage are members of the CCAAT/enhancer binding protein (C/EBP) family. The beta and gamma subtypes (C/EBPβ and C/EBPγ) are expressed before the cell cycle arrest phase and appear to stimulate the expression of genes required for this pre-requisite (Hwang *et al.*, 1997). In particular, *in vivo* experiments have identified that C/EBPβ up-regulates the mRNA expression of peroxisome proliferator activated receptor gamma (PPARγ) (Wu *et al.*, 1995). Binding sites for C/EBPβ have been identified in the promoter regions of PPARγ as well as C/EBPα (Tang *et al.*, 2004), which have both been shown to be involved with cell cycle arrest (Umek *et al.*, 1991, Altiok *et al.*, 1997). Studies have shown that adipocyte differentiation in the presence of C/EBPα does not occur if PPARγ is not also present (Rosen *et al.*, 2002). However, differentiation does occur in the presence of PPARγ without C/EBPα, resulting in PPARγ being branded the master regulator of adipogenesis. PPARγ, largely specific to adipose tissue, is a nuclear transcription factor that is highly expressed in adipose cells early in development. It acts to promote the expression of other adipogenic genes by forming heterodimeric complexes with the retinoid X receptor (RXR) which interact directly with DNA regions. Another key

adipogenic gene expressed during early adipogenesis is sterol regulatory element-binding protein variant 1c (SREBP-1c) which stimulates the expression of many genes required for lipogenesis (Horton *et al.*, 2002). The ability of adipocytes to carry out lipogenesis and accumulate lipid signifies the final phase of maturation. During this time, the mRNA expression of genes involved in lipogenesis and fatty acid uptake, including fatty acid synthase (FAS), lipoprotein lipase (LPL), glycerol-3-phosphate dehydrogenase (GPDH), stearoyl-CoA desaturase (SCD) as well as others, increases drastically (Gregoire *et al.*, 1998). Mature adipocytes become increasingly sensitive to insulin through up-regulation of glucose transporters and insulin receptors (Garcia de Herreros and Birnbaum, 1989) and begin to synthesise adipokines such as leptin and adiponectin (Gregoire *et al.*, 1998).

1.4.2.2 Regulation of adipogenesis by hormones and growth factors

Studies have shown that the cascade of events that leads to the eventual maturation of adipocyte cells is sensitive to signals from growth factors and hormones. Observational studies in humans have shown an association between growth hormone (GH) and increased body fat mass. This has led to the hypothesis that GH plays a key role in adipogenesis which has been supported by studies that confirmed the ability of GH to induce differentiation *in vitro* (Morikawa *et al.*, 1982). GH is also known to stimulate gene expression of insulin-like growth factor-1 (IGF-1) which is necessary for adipocyte differentiation (Smith *et al.*, 1988). IGF-1 receptors are found on pre-adipocytes and their abundance increases during adipocyte maturation resulting in the increased sensitivity of maturing adipocytes to insulin and IGF-1. Binding of insulin and IGF-1 to their receptors triggers an intracellular signalling pathway which results in adipocyte differentiation (Kamai *et al.*, 1996). Many other factors have been demonstrated to stimulate (glucocorticoids, PGE_2 and PGI_2) or suppress (EGF, TGFα, TGFβ) differentiation (Gregoire *et al.*, 1998) leading to a complex regulation of the formation of new adipocytes. It is also important to note, however, that the aggregation of large adipose tissue stores is not just as a result of increased adipocyte number (hyperplasia), but is in fact primarily due to an increase in cell size (hypertrophy).

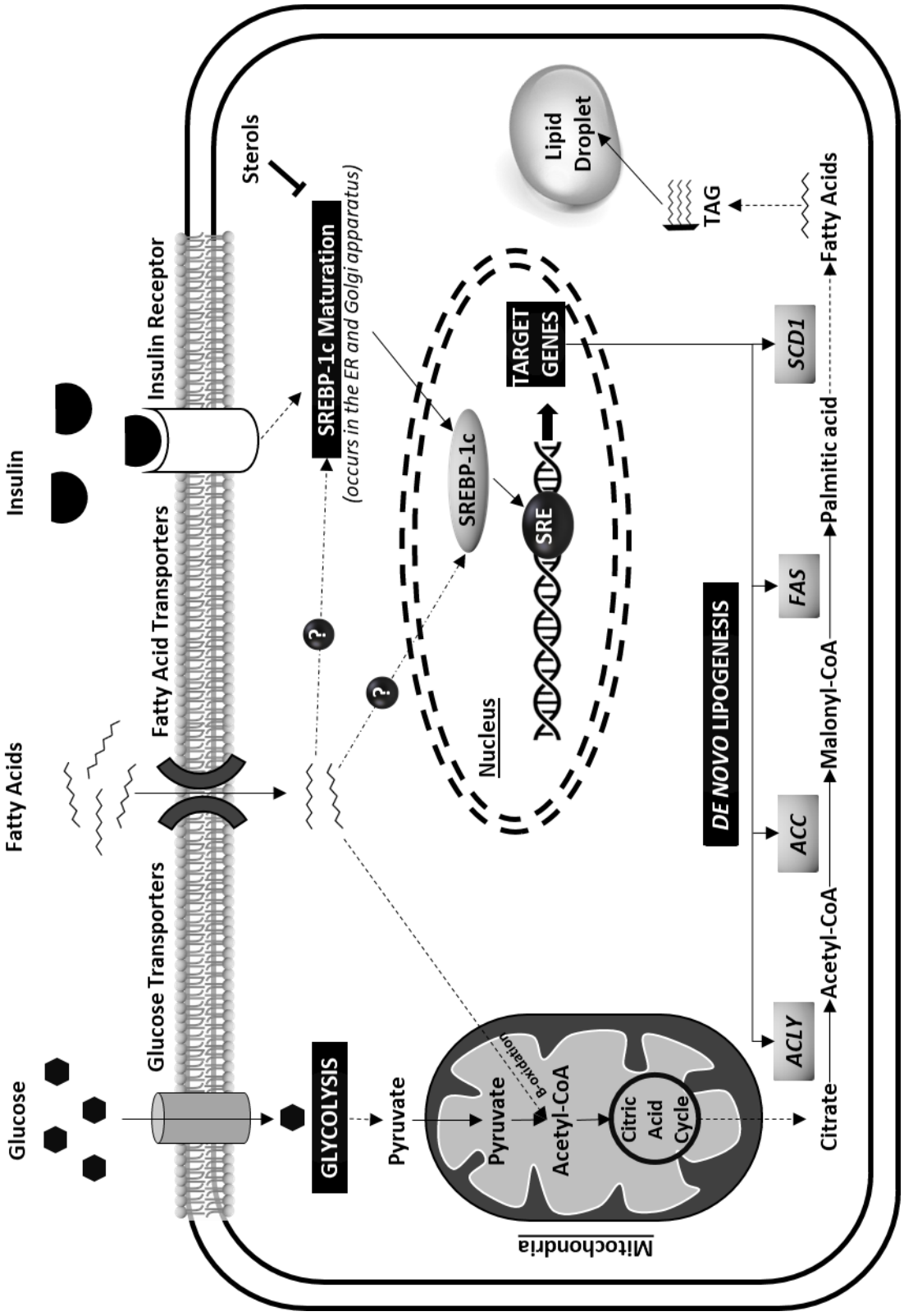
1.4.3 Lipogenesis and lipolysis

Whilst the formation of new fat cells revolves around the differentiation of pre-adipocytes, the amount of fat stored within a mature adipocyte is dependent on three processes: (1) lipid synthesis or lipogenesis, (2) lipid breakdown or lipolysis and (3) fatty acid uptake from the circulation. Fatty acids are essential cellular constituents with critical roles in signalling, energy supply and cell structure. They are the functional units of fat and are stored within adipocytes within TAG molecules, a glycerol backbone bound to three fatty acids. Lipogenesis refers to the formation of TAG from fatty acids consumed within the diet and/or produced endogenously. The latter is more specifically termed *de novo* lipogenesis and utilises carbohydrates as a source of glucose which is then converted into fatty acids in a multistep pathway summarised in Figure 1.3. Lipolysis refers to the breakdown of TAG into glycerol and free fatty acids which can then be further broken down to yield energy in the form of ATP in a process termed β-oxidation. When the rate of lipogenesis exceeds the rate of lipolysis then lipid storage, and hence fat mass, increases. Lipogenesis, predominantly occurring in adipose tissue but also in the liver, skeletal muscle, heart and pancreas, is under precise control by many metabolic and hormonal factors.

De novo lipogenesis is chiefly under the enzymatic control of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Volpe and Vagelos, 1976, Bloch and Vance, 1977). These enzymes act on acetyl-CoA and malonyl-CoA respectively to produce palmitic acid which can then be stored or used to produce other fatty acids. Many enzymes involved in *de novo* lipogenesis are under the transcriptional control of sterol regulatory element binding proteins (SREBP) of which there are three-subtypes: SREBP-2, SREBP-1a and SREBP-1c. SREBP-1c has been shown to be the dominant isoform associated with lipogenesis (Horton *et al.*, 2002) and activates many lipogenic genes (Figure 1.3).

Figure 1.3 (Overleaf) Overview of *de novo* lipogenesis (DNL) which occurs in the cytoplasm of most cells, particularly adipocytes and hepatocytes. Fatty acids are produced via the synthesis of palmitic acid from metabolites produced in the citric acid cycle. Dietary glucose and/or fatty acids are transported into cells via their respective transporters where they can enter the citric acid cycle. Citrate is converted in acetyl-CoA by ATP citrate lyase (ACLY). The two central enzymes responsible for DNL,

acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), then further synthesise palmitic acid (C16:0). A range of fatty acids can then be made from palmitic acid through multi-step processes requiring various different enzymes, one of which is the rate limiting enzyme Stearoyl-CoA desaturase-1 (SCD1) which is responsible for the production of monounsaturated fatty acids. The enzymes involved in DNL are under the transcriptional control of sterol regulatory element-binding protein 1 (SREBP-1c) due to the presence of SREBP response elements (SRE) in the promotor regions of these genes. The biological activity of SREBP-1c relies on its maturation and release from the membranes of the endoplasmic reticulum (ER) and the Golgi apparatus. It is well understood that this process is inhibited by sterols such as cholesterol, however, insulin (Soyal *et al.*, 2015) as well as some fatty acids (Xu *et al.*, 2001) are also thought to regulate this process. (Adapted from Song *et al.* (2018)).


Glucose
Glucose Transporters
Fatty Acids
Fatty Acid Transporters
Insulin
Insulin Receptor
Sterols
SREBP-1c Maturation
(occurs in the ER and Golgi apparatus)
Lipid Droplet
TAG
Fatty Acids
TARGET GENES
SCD1
SREBP-1c
SRE
Nucleus
?
?
DE NOVO LIPOGENESIS
FAS
Palmitic acid
ACC
Malonyl-CoA
ACLY
Acetyl-CoA
Citrate
GLYCOLYSIS
Pyruvate
Pyruvate
B-oxidation
Acetyl-CoA
Citric Acid Cycle
Mitochondria

1.5 An introduction to dietary fatty acids

1.5.1 Types of dietary fatty acids

Fatty acids are carboxylic acids consisting of a hydrocarbon chain of varying length and a terminal carboxyl group. Fatty acids mainly exist in an esterified form such as those found in oils, animal fats and waxes and they can be classified based on the number of double bonds within the hydrocarbon chain. Saturated fatty acids (SFA) contain no double bonds and are found in animal products such as meat and dairy, as well as some tropical oils (e.g. coconut and palm oil). Monounsaturated fatty acids (MUFA) contain a single double bond and are found in high quantities in olive oil, macadamia oil and a number of other nuts and seeds. Polyunsaturated fatty acids (PUFA) contain more than one double bond. PUFA are further subcategorised into omega-3, omega-6 and omega-9 families based on the location of the double bonds and it is becoming increasingly clear that individual fatty acids within their broad classifications carry unique biological characteristics and health effects.

Whilst all three PUFA families carry biological importance, it is the omega-3 and omega-6 families that are of greatest interest to research as they are considered essential fatty acids (Burr and Burr, 1930). Specifically the 18 carbon omega-6 family precursor linoleic acid (LA), found in high amounts in soy, corn, safflower and sunflower oils, and the omega-3 family precursor alpha-linolenic acid (ALA), found in leafy green vegetables as well as flaxseed and canola oil, cannot be synthesised within the human body and must be consumed in the diet and failure to do so is fatal (Burr and Burr, 1930).

1.5.2 Setting the scene for dietary PUFA

It is well established that poor dietary habits result in excessive weight gain in children and adults. A key characteristic of obesity is increased central and visceral adiposity, and dietary fats are often implicated as a key driver. It is clear, however, that not all dietary fats are equal in their biological structure and function and so some may contribute to obesity and its associated health outcomes more than others. Efforts have been made to identify those components in an attempt to address the obesity epidemic on a population level.

Obesity is a major risk factor for CVD (see section 1.1) and early studies found an association between circulating cholesterol levels and risk of death from CHD. A study by Keys (1970) also found a strong correlation between dietary intake of saturated fat and blood cholesterol concentrations (Keys, 1970). At around the same time, dietary PUFA were shown to reduce cholesterol levels (Keys *et al.*, 1965). This resulted in the hypothesis that SFA were a key driver of CVD and consequently governments recommended that SFA in the diet, particularly animal fats, be replaced with PUFA (vegetable oils) in order to decrease the risk of CHD. For the UK, as well as other developed countries, this led to a substantial decrease in animal fat consumption in the last 40 years (SACN, 2019). Interestingly, however, the dramatic reduction in the consumption of SFA, and corresponding increase in the intakes of PUFA, at a population level was not associated with any reduction in the deaths from CVD. In fact, ischaemic heart disease still remains the leading cause of death worldwide, particularly in middle- and high-income countries where such government level recommendations were made (WHO, 2017b). Regardless of this, saturated fat continues to be regarded as a potential cause of the increased rates of CVD in populations worldwide, despite evidence that some SFA exert cardio-protective effects. Dairy products such as cheese have been labelled as harmful due to their high saturated fat content despite having relatively high levels of monounsaturated fats and despite studies that show some level of cardio-protection associated with higher levels of cheese consumption (Gholami *et al.*, 2017). There is little doubt that cholesterol levels can be reduced in some individuals by substituting saturated fats with PUFA, however, this does not always translate to reductions in rates of death from CVD (Ramsden *et al.*, 2013c) and this topic needs to be continually reassessed. However, despite these new insights and re-evaluation of the role of saturated fat within the diet, dietary patterns over the past 60 years have shifted dramatically in Western countries towards a reduction in saturated fat and an increase in dietary PUFA.

1.5.3 Current recommendations and intakes of dietary PUFA

1.5.3.1 Recommended intakes

Broad government recommendations to replace SFA with PUFA in the diet have led to a population-level shift in the fatty acid composition of Western diets such as those consumed in the UK, USA and Australia. Primarily this shift has been caused by the

replacement of butter and other saturated fats (e.g. lard) with plant-based cooking oils such as canola and sunflower oils, which are high in LA, the omega-6 precursor. Whilst this replacement successfully reduced the SFA intake within the diet (WHO and FAO, 1994), the use of PUFA as an umbrella term can be misleading since different families, and indeed individual fatty acids, can exhibit quite different biological roles (see section 1.5.4.1). Whilst the recommended intakes of dietary PUFA vary between countries (Ailhaud *et al.*, 2006), a joint report from the FAO and WHO (2008) recommended that total omega-6 intake represents 2.5-9% energy (%en) of the diet whilst total omega-3 represents 0.5-2%en, translating to a ratio of ~5:1 for total-omega-6 and total omega-3 respectively. The report also suggested that the intake of long chain polyunsaturated fatty acid (LCPUFA) derived from omega-3 (i.e. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) should be increased for pregnant and lactating women, no increase in any omega-6 fatty acids were suggested. The minimum values in this report are based on the avoidance of deficiency symptoms, whilst the maximum values were set in accordance with research highlighting the capacity for LCPUFA, particularly omega-3 LCPUFA, to reduce CVD risk. It is therefore apparent that many of the biological benefits associated with omega-3 PUFA are as a result of conversion to longer chain derivatives. Some studies in animals have shown that a LA:ALA ratio of <5:1 is most appropriate for enhanced omega-3 LCPUFA synthesis and accumulation in tissues (Blank *et al.*, 2002, Gibson *et al.*, 2011). Other studies have further specified that maximal conversion of ALA to EPA and DHA occurs when the dietary ratio of LA:ALA is set at 1-2:1 but overall intake of dietary PUFA is low (Tu *et al.*, 2010). It is suggested that this dietary ratio is associated with CVD mortality at one-fourth the current rate in the USA (Lands, 2000).

1.5.3.2 Estimated intakes

Increasing the use of vegetable oils has resulted in a dramatic increase in the intake of omega-6 PUFA, with LA being the most abundant fatty acid consumed in a Western diet (Ramsden *et al.*, 2013c, Wood *et al.*, 2014). This increase in omega-6 consumption has not been matched by an increase in omega-3 which has remained stable over this period of dietary change (Figure 1.4) (Ailhaud *et al.*, 2006). The ratio of omega-6 to omega-3 in current Western diets is reported to be around 8-10:1 (Blasbalg *et al.*, 2011, Meyer *et al.*, 2003) or higher (Simopoulos, 2016). This is higher than

recommendations and studies investigating the rate of conversion to long chain derivatives. The consequence of this high omega-6 to omega-3 ratio of dietary PUFA for public health is unclear.

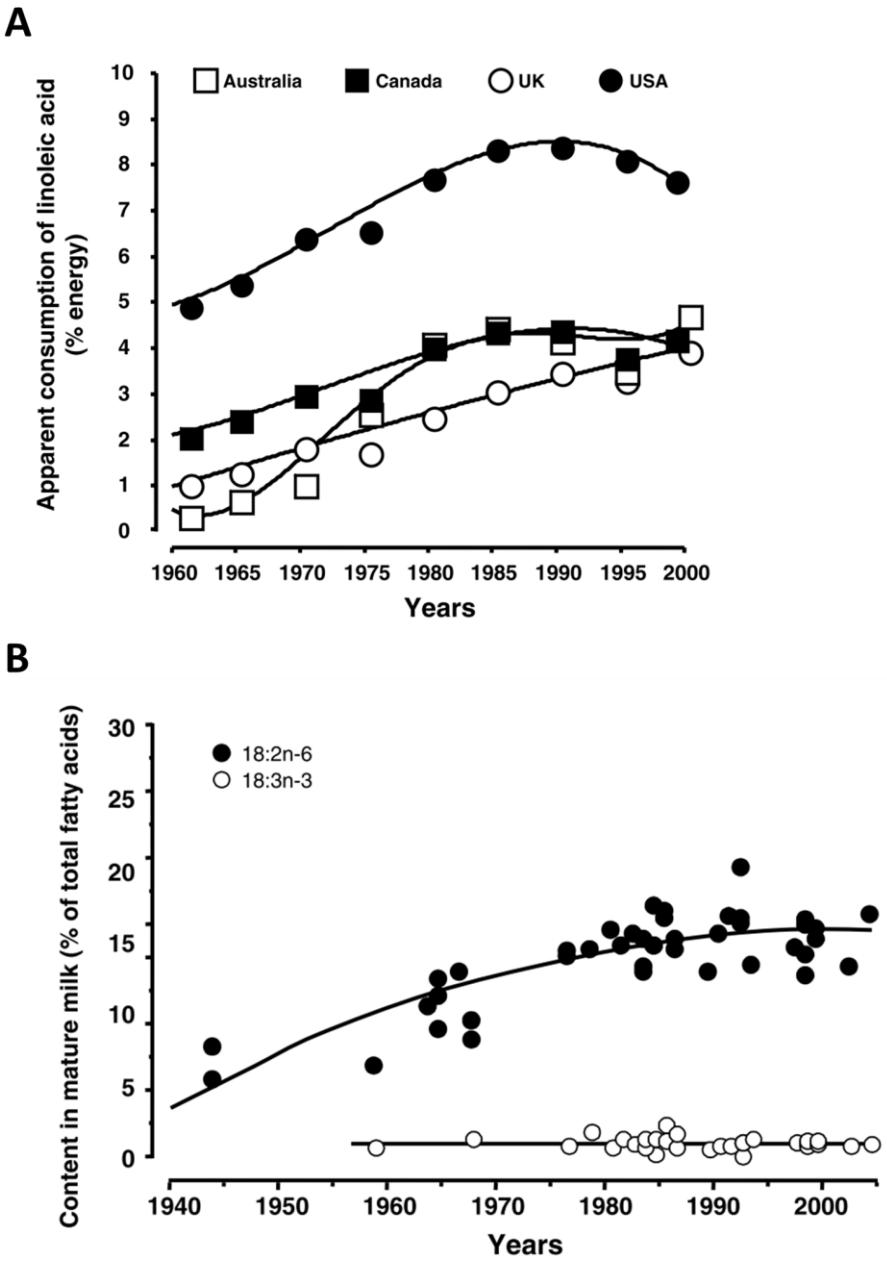


Figure 1.4 Temporal changes in (A) apparent consumption of linoleic acid in Western countries and (B) linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) content in mature breast milk of US women (taken from Ailhaud *et al.* (2006)).

1.5.4 Omega-6 and omega-3 PUFA

1.5.4.1 Metabolism and function

The biological functions of the omega-6 and omega-3 classes are primarily undertaken by the long-chain derivatives of the parent compounds, LA and ALA, and so there is a requirement for them to be converted to these derivatives in order to mediate many of their biological effects. This multistep process, established by Holman (1986) and further refined by Sprecher *et al.* (1995), relies on the addition of carbon molecules to increase chain length (elongation) and the addition of double bonds within the carbon chain (desaturation). As summarised in Figure 1.5, LA (18:2 n-6) and ALA (18:3 n-3) are converted to 18:3 n-6 and 18:4 n-3 respectively by the enzyme Δ6 desaturase encoded by the *Fads2* gene. These desaturation products are further converted into arachidonic acid (AA; 20:4 n-6) and eicosapentaenoic acid (EPA; 20:5 n-3) respectively through the action of Δ5 desaturase, encoded by the *Fads1* gene, and a series of elongation steps. EPA is further converted into docosapentaenoic acid (DPA; 22:5 n-3) via elongation. A further desaturation, which utilises Δ6 desaturase once more, and a final β-oxidation step produces docosahexaenoic acid (DHA; 22:6 n-3). It is these longer chain fatty acids that carry important biological roles.

Both omega-3 and omega-6 PUFA are involved in many important biological functions, demonstrated by the absolute requirement of LA and ALA in the diet. They are an important constituent of cell membranes (Spector, 1999) and so influence cell membrane fluidity as well as the behaviour of membrane bound enzymes and receptors (Buettner *et al.*, 2007). They also play a role in controlling blood pressure and are key to proper brain and neural development (Wall *et al.*, 2010). The long chain derivatives of both families, AA in the omega-6 pathway and EPA and DHA in the omega-3 pathway, also have important roles in inflammation and immune regulation through the actions of their own derivatives known as eicosanoids (Calder, 2006, Patterson *et al.*, 2012).

1.5.4.2 Eicosanoids and their role in inflammation

Eicosanoids are biologically active lipids and include a variety of molecules which generally fall into the subcategory of prostaglandins (PG), thromboxanes (TX),

leukotrienes, resolvins or lipoxins. Eicosanoids are derived from 20-carbon fatty acids (such as AA and EPA) which are liberated from the plasma membrane by phospholipases (Figure 1.5). Eicosanoids are then metabolised through the sequential actions of various enzymes including cyclooxygenases (COX) and lipoxygenases (LOX). Metabolism of AA by the COX enzymes results in the synthesis of 2-series prostaglandins (PGE_2, PGI_2, PGD_2 and $PGF_{2\alpha}$) and thromboxanes (TXA_2 and TXB_2) and 4-series leukotrienes (Patterson *et al.*, 2012). When EPA is used as a substrate for COX and LOX enzymes, as opposed to AA, prostaglandins and thromboxanes of the 3-series and the 5-series of leukotrienes are produced (Patterson *et al.*, 2012).

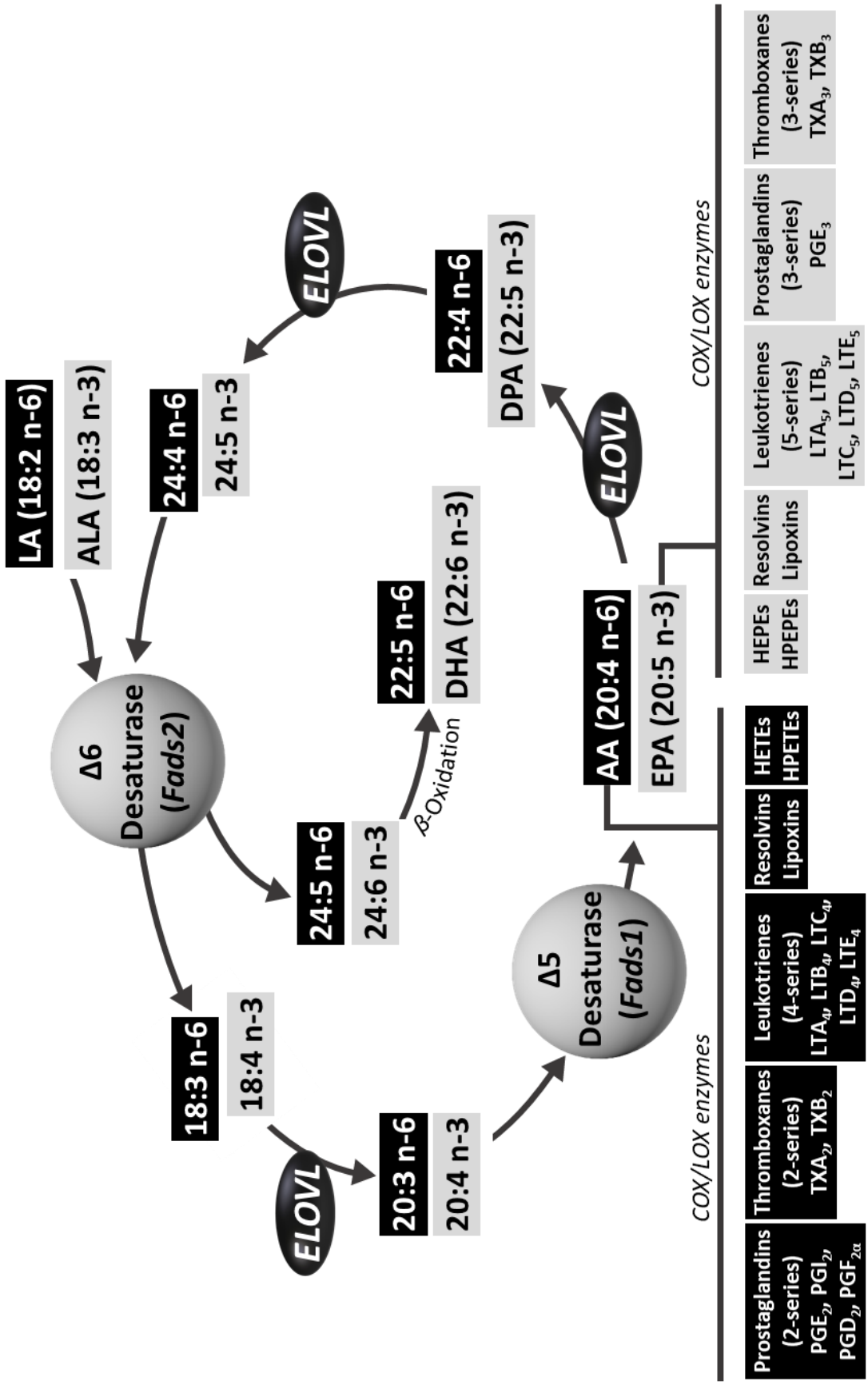
Eicosanoids play a critical role in many processes involved in inflammation, which in healthy individuals facilitates a natural bodily defence against infection and injury (Ricciotti and FitzGerald, 2011). Inflammation of this kind is caused by induction of a pro-inflammatory signalling cascade that is rapidly mediated by a host of anti-inflammatory mediators acting through a negative feedback mechanism resulting in an acute inflammatory response, with relatively rapid resolution. However, when these regulatory mechanisms falter, or there is an imbalance in pro-inflammatory and anti-inflammatory signals, excessive and ongoing inflammation can occur (Calder, 2015). As such, the dysregulation of eicosanoid production has been implicated in diseases with an inflammatory component such as CVD, obesity, inflammatory bowel diseases, rheumatoid arthritis and cancer (Patterson *et al.*, 2012).

Eicosanoids derived from AA and EPA have varied properties, both pro-inflammatory and anti-inflammatory in nature and all play an important role in the regulation of inflammation. However, it is generally considered that eicosanoids derived from AA are more pro-inflammatory in nature whilst those derived from EPA are more anti-inflammatory. PGI_2 and PGE_2, generated from AA, have pro-arrhythmic effects whereas PGI_3 and PGE_3 are anti-arrhythmic (Li *et al.*, 1997). Similarly, AA derived TBX_2 is a potent vasoconstrictor and platelet activator whereas TBX_3, derived from EPA, has few physiological effects. AA derived eicosanoids also activate the production of inflammatory cytokines, including TNF_α, IL-1 and IL-6 (Schmitz and Ecker, 2008). Resolvins derived from EPA (E-series) and DHA (D-series) also exert anti-inflammatory effects by suppressing the activation of $NF_K\beta$, resulting in

decreased synthesis of inflammatory cytokines, a process that appears to be dependent on PPARγ activation (Liao *et al.*, 2012).

EPA, DHA and AA are competitive substrates for enzymes involved in the synthesis of eicosanoids and diets high in omega-3 PUFA have been shown to decrease production of PGE_2, TXB_2, TNF_α and IL-1 in humans (Caughey *et al.*, 1996). The utilisation of either AA or EPA/DHA as a precursor for eicosanoid production relies on their synthesis and incorporation into cell membranes. The formation of the LCPUFA from LA and ALA utilises the same enzymes for elongation and desaturation (Figure 1.5). This sharing of enzymes for this multistep process results in competition between the two families for substrate binding and thus, a differing ratio of dietary precursors can lead to an imbalanced production of long chain derivatives and hence a more pro-inflammatory or anti-inflammatory eicosanoid profile.

Figure 1.5 (Overleaf) The metabolism of dietary linoleic acid (LA) and alpha-linolenic acid (ALA) to long-chain fatty acid derivatives and eicosanoid biosynthesis (adapted from Patterson *et al.* (2012) and Gibson *et al.* (2011)).


LA (18:2 n-6)
ALA (18:3 n-3)
Δ6 Desaturase (Fads2)
18:3 n-6
18:4 n-3
ELOVL
20:3 n-6
20:4 n-3
Δ5 Desaturase (Fads1)
AA (20:4 n-6)
EPA (20:5 n-3)
24:4 n-6
24:5 n-3
ELOVL
24:5 n-6
24:6 n-3
β-Oxidation
22:5 n-6
DHA (22:6 n-3)
22:4 n-6
DPA (22:5 n-3)
ELOVL
COX/LOX enzymes
COX/LOX enzymes
Prostaglandins (2-series) PGE2, PGI2, PGD2, PGF2α
Thromboxanes (2-series) TXA2, TXB2
Leukotrienes (4-series) LTA4, LTB4, LTC4, LTD4, LTE4
Resolvins Lipoxins
HETEs HPETEs
HEPEs HPEPEs
Resolvins Lipoxins
Leukotrienes (5-series) LTA5, LTB5, LTC5, LTD5, LTE5
Prostaglandins (3-series) PGE3
Thromboxanes (3-series) TXA3, TXB3

1.5.5 Enzymes and competition between families

1.5.5.1 Desaturase and elongase enzymes

Desaturase enzymes *Fads1* and *Fads2* are found in a variety of tissues but are present at highest levels in the liver. They are responsible for the addition of double bonds to fatty acids in the LCPUFA synthesis pathway (Figure 1.5). *Fads2* is considered a key regulatory enzyme in the synthesis of LCPUFA as it is used twice in the conversion of ALA to DHA (Sprecher *et al.*, 1995). The importance of the *Fads2* gene in synthesis of LCPUFA has been clearly demonstrated in mice as *Fads2* knockout mice lack the ability to carry out the initial step of LCPUFA synthesis which results in downstream consequences for PUFA metabolites manifesting in sterility in both males and females (Stoffel *et al.*, 2008, Stroud *et al.*, 2009). Stroud *et al.* (2009) also demonstrated the importance of the *Fads1* gene in LCPUFA synthesis with radiolabelled administration of LA and ALA. *Fads2* knockout mice accumulated levels of 20:3 n-6 and 20:4 n-3, fatty acid metabolites produced from LA and ALA respectively. This suggests that *Fads1* has some desaturation capacity when expression of *Fads2* is suppressed.

Fatty acid elongation is carried out by the elongation of very long chain fatty acids proteins (ELOVL), of which 7 subtypes have been identified in humans and rodents. ELOVL2 and ELOVL5 are the key elongase enzymes associated with LCPUFA synthesis in mammals and are expressed in high amounts in the liver (Jakobsson *et al.*, 2006). Knockout experiments have highlighted that the initial elongation step is dependent on ELOVL5 but increased activity of ELOVL2 in ELOVL5 knockout mice also suggests a compensatory mechanism whereby ELOVL2 acts to ensure LCPUFA synthesis (Moon *et al.*, 2009). Interestingly, mRNA expression of SREBP-1c was up-regulated in ELOVL5 knockout mice due to the decreases in cellular concentrations of DHA and AA. This up-regulation led to the up-regulation of SREBP-1c target genes responsible for lipogenesis which consequently resulted in hepatic steatosis i.e. fat accumulation in the liver (Moon *et al.*, 2009).

The expression of the desaturase and elongase enzymes have been shown to be sensitive to dietary intakes. *In vivo* and *in vitro* studies have demonstrated that both *Fads1* and *Fads2* mRNA expression and enzyme activity are suppressed in the presence of adequate omega-3 and omega-6 dietary PUFA and elevated when dietary

PUFA is low (Cho *et al.*, 1999a, Cho *et al.*, 1999b, Tu *et al.*, 2010). Expression of ELOVL5 is also sensitive to nutritional status such that high levels of omega-3 suppress mRNA expression, however, ELOVL2 expression does not appear to be regulated by dietary fat intake (Wang *et al.*, 2005).

1.5.5.2 Pathway competition

Animal studies have demonstrated that, if they are not directly consumed in the diet, the abundance of the 20-carbon fatty acids (i.e. AA and EPA) is determined by the ratio of their precursors, LA and ALA in the diet. Blank *et al.* (2002) demonstrated that in piglets fed a varying LA:ALA ratio the predominant LCPUFA in plasma phospholipids of those receiving a low LA:ALA ratio was EPA and in those receiving a high LA:ALA ratio was AA. Similar observations were made in the livers of rats receiving diets containing increasing amounts of ALA (%en) and thus a decreasing LA:ALA ratio. A dose-response relationship was observed such that EPA levels increased as the LA:ALA ratio in the diet decreased (Tu *et al.*, 2010). This has also been demonstrated in humans where decreasing dietary intakes of LA, with no changes in omega-3 intake, resulted in an increase in omega-3 LCPUFA in plasma phospholipids (Wood *et al.*, 2014). These studies highlight that a LA dominant diet results in AA as the major 20-carbon fatty acid in tissues and accumulation of EPA can only occur when the LA:ALA ratio of diets is low.

This imbalance in LCPUFA synthesis can partially be explained by simple substrate competition. Brenner and Peluffo (1966) demonstrated an inverse relationship between the conversion of LA to 18:3 n-6 and the amount of ALA added, implying that ALA and LA compete for the same desaturation enzyme and this is likely to be at the initial step where LA and ALA utilise *Fads2*. This same study also demonstrated that the *Fads2* enzyme had a higher affinity for ALA compared to LA adding further complication to the competition between these two substrates. This suggests that conversion of omega-3 fatty acids to LCPUFA is preferential over conversion of omega-6 fatty acids and by lowering the amount of omega-6 substrate, i.e. LA, the amount of omega-3 LCPUFA being synthesised would increase. The competition between LA and ALA for conversion to their longer-chain derivatives was also demonstrated in a study by Clark *et al.* (1992), in which they lowered the LA:ALA ratio of formula milk (19:1) by either increasing the amount of ALA (4:1) or

decreasing the amount of LA (3:1). Decreasing the LA:ALA ratio by either means was associated with an increased incorporation of omega-3 LCPUFA into erythrocytes when compared with formula milk. This further highlights the importance of the LA:ALA ratio in determining the LCPUFA status of humans but also demonstrated that reducing the amount of LA in the diet is a practical method for increasing omega-3 LCPUFA status.

That the pathway utilises the *Fads2* enzyme twice in the synthesis of LCPUFA suggests another potential competition point between fatty acids for enzyme substrate activity (Figure 1.5). A linear relationship between substrate supply, i.e. LA and ALA, and accumulation of 20-carbon chain fatty acids, AA and EPA, has been demonstrated in pigs (Blank *et al.*, 2002), rats (Tu *et al.*, 2010) and repeatedly in human studies (Wood *et al.*, 2015, Mantzioris *et al.*, 1994, Chan *et al.*, 1993). However, what these studies, as well as others (Cleland *et al.*, 2005), have also highlighted was that the relationship between the dietary intake of LA and ALA and circulating and tissue accumulation of DHA was more complicated. Increasing dietary levels of ALA increased levels of DHA in a curvilinear fashion such that DHA accumulation was maximal when dietary ALA was at 1-2%en (Tu *et al.*, 2010, Blank *et al.*, 2002). As such, it has been postulated that competition exists between the first step of LCPUFA synthesis and the final steps to produce DHA such that DHA production is limited when dietary ALA is high (Mantzioris *et al.*, 1994, Chan *et al.*, 1993).

Further to this, fatty acids of both families rely on incorporation into cell membranes to carry out many of their biological functions. A study in non-human primates fed preformed EPA to isolate and investigate competition at the incorporation stage as opposed to synthesis stage. This study found that EPA incorporation into erythrocyte phospholipids was greatest when dietary LA was reduced (McMurchie *et al.*, 1990), thus, suggesting another point of competition between the two families of fatty acids.

These studies highlight the complexity of the interactions between fatty acids and enzymes in this pathway and the importance of the dietary LA:ALA ratio as well as the overall fat and PUFA content of diets in determining the synthesis of omega-3 LCPUFA, and their incorporation into tissues.

1.6 Fetal requirements for fatty acids and lipids

Both omega-3 and omega-6 fatty acids are required by the developing fetus for the maintenance of cell membrane fluidity and permeability, as precursors of important bioactive molecules and as a key source of energy.

Fat deposition within the fetus increases dramatically with gestational age in humans and indeed, some of the fatty acids deposited in adipose tissue will be generated through fetal lipogenesis (Cetin *et al.*, 2009). The majority of the fatty acids in fetal lipid, however, and all of the omega-6 and omega-3 fatty acid precursors, are derived from the maternal circulation. Transfer of these fatty acids from maternal to fetal blood supply requires transport across the placenta. The placenta, as well as the fetal liver during early gestation, has been shown to lack the necessary enzymes to convert LA and ALA to their longer chain derivatives (Mercuri *et al.*, 1979b). As such, it can be assumed that fetal supply of both the omega-6 and omega-3 precursors as well as their longer chain derivatives is dependent on the levels of these factors in the maternal circulation, and hence in the maternal diet. This could lead one to expect that fetal PUFA concentrations would mimic that of the mother. It has been shown, however, that concentrations of AA and DHA are higher in all of the major lipid classes in the fetus when compared to maternal blood, a process termed biomagnification (Haggarty *et al.*, 1999, Larque *et al.*, 2003). This suggests a level of preferential transfer of some fatty acids over others. Perfusion studies have identified such a hierarchy and placental preference for uptake of fatty acids is as follows AA>DHA>ALA>LA>oleic. AA, however, is preferentially retained by the placenta for prostaglandin synthesis (Kuhn and Crawford, 1986) and so the hierarchy of fatty acids transferred to fetal circulation therefore becomes DHA>ALA>LA>oleic>AA (Haggarty *et al.*, 1997).

The fact that DHA concentrations are higher in the fetal circulation compared to the maternal circulation, as well as the finding that this fatty acid is preferentially transferred across the placenta in comparison to other fatty acids, suggests an increased fetal requirement and hence important biological function of this fatty acid in fetal development. The critical role of omega-3 fatty acids, in particular DHA, during fetal development was first established in studies on non-human primates. Neuringer *et al.* (1986) demonstrated that mothers consuming an omega-3 deficient diet gave birth to offspring with impaired cognitive function and visual acuity which could not be

corrected by omega-3 supplementation in later life. This was supported by the finding that the fetal brain and retinal phospholipids incorporate DHA ten times faster than LA and ALA (Cetin *et al.*, 2009).

1.7 PUFA and associated disease risk

1.7.1 Dietary PUFA and disease risk in adults

The increasing intakes of LA in the Western diet (Figure 1.4), coupled with the pro-inflammatory action of omega-6 fatty acids, has led to the hypothesis that LA may be contributing to the upwards trends in obesity, metabolic syndrome and risk of CVD in human populations worldwide.

1.7.1.1 Cardiovascular diseases

Human studies on patients at risk of coronary artery disease (CAD) found that supplementation with omega-3 LCPUFA led to a reduction in mortality from CAD and CVD (Dolecek and Granditis, 1991), as well as a reduction in cardiac events (Singh *et al.*, 1997), when compared to control groups. Similarly, dietary omega-3 intake was associated with decreased risk of CHD and CHD mortality in a group of 80000 female nurses (Hu *et al.*, 2002). However, the cardio-protective properties of omega-3 LCPUFA have not been demonstrated in all studies, with several large scale robust randomised control trials finding no effect of omega-3 supplementation on cardiovascular events in high (Bosch *et al.*, 2012, Bowman *et al.*, 2018) and average risk patients (Manson *et al.*, 2018).

While these recent findings have generated a degree of controversy about the role of omega-3 fatty acids in the prevention and treatment of CVD, a report released by the FAO and WHO (2008) still concluded that there was convincing evidence that omega-3 LCPUFA may decrease the risk of death from CHD. The report does not suggest there are data linking increased intake of LA to CVD outcomes despite evidence from the Sydney Diet Heart Study (SDHS) where saturated fats were replaced with concentrated sources of LA, and an association between the dietary intervention and increased risk of death from CHD was observed (Ramsden *et al.*, 2013b). However, with the established complexity of the interactions and competition between omega-3 and omega-6 fatty acids, it may be naïve to look at their effects on disease risk in

isolation as consumption of one family undoubtedly affects the metabolism and functional capacity of the other.

1.7.1.2 Obesity

The above text has discussed the capacity for AA to produce pro-inflammatory metabolites, however, some of these have also been shown to be pro-adipogenic. The metabolites 9-HODE and 13-HODE increase PPARγ expression resulting in increased adipocyte differentiation. Similarly, 20-HETE can induce oxidative stress associated with increased adiposity through its ability to up-regulate adipocyte differentiation (Kim *et al.*, 2013). AA has also been shown to be capable of stimulating adipogenesis and lipogenesis *in vitro* (Massiera *et al.*, 2003). This capacity appears to be mediated via the conversion of AA to PGI_2 which is a known ligand of PPARβ/δ and capable of stimulating adipogenesis through PPARγ activation (see section 1.4). Omega-6 PUFA have also been shown to affect the expression of key lipogenic genes. A study by Muhlhausler *et al.* (2010a), where rats were fed diets containing increasing levels of ALA against a consistent LA content, showed a positive correlation between the adipose tissue expression of SREBP-1c and FAS and erythrocyte omega-6 PUFA concentrations. Increased expression of these genes suggests an increase in lipogenic capacity of these tissues and hence increased lipid storage which may contribute to obesity.

These *in vivo* animal studies highlight the capacity for a role of LA in the pathogenesis of obesity, however, at a population level this link remains controversial. It is difficult to isolate the effects of single nutrients in human studies and appropriately take into consideration lifestyle, genetic and dietary factors that contribute to the development of obesity. Some studies have attempted to investigate this and a study on German men and women found an association between LA intake and weight gain in women over a 6.5 year period (Nimptsch *et al.*, 2010). Whilst this study relied on the accurate self-reporting of food frequency via questionnaires, Wang *et al.* (2016) found a correlation between baseline erythrocyte LA levels and weight gain resulting in overweight or obesity after a 10 year follow-up. Using erythrocyte LA levels as a proxy for intake addresses the criticisms for relying on self-reported intakes.

1.7.2 Dietary PUFA and disease: A DOHaD perspective

Whilst there is some characterisation of the effects of omega-3 and omega-6 PUFA in adults, less is known about how this might impact on the programming of disease in the next generation. The prevalence of obesity in children is increasing at an alarming rate. Given that obesity is believed to track from childhood to adulthood this may predispose individuals to adverse health issues in later life. The nutritional environment that an individual is exposed to during development has been shown to affect growth and fat deposition as well as disease risk in later life (see section 1.2). In particular, the effects of a maternal diet high in fat are relatively well characterised in animal models and are typically associated with a range of offspring phenotypes, including high blood pressure, increased adiposity and impaired insulin and glucose homeostasis. With obesity being a major risk factor for metabolic syndrome, type 2 diabetes and risk of CVD, identifying possible contributors and windows for prevention of the occurrence of obesity is a major global priority. Obesity is characterised by an increase in lipid storage and as a chronic inflammatory state. The biological actions of LA and its derivatives (see section 1.5.4.2) as pro-adipogenic and pro-inflammatory, their ability to affect genes involved in lipogenesis and evidence from studies in adults, all suggest a link between the intake of LA and obesity and may provide a clear biological basis for the hypothesis that current dietary trends are contributing to the intergenerational cycle of obesity.

1.7.2.1 Animal studies

Animal studies have provided the fundamental basis for exploring this hypothesis. Massiera *et al.* (2003) fed a diet rich in LA to mice during pregnancy and lactation. The adult progeny exhibited increased weight and adiposity compared to those fed a diet with lower levels of LA alongside a dietary source of ALA. Interestingly, when PGI_2 receptor knockout mice were fed the same diets, no difference in body weight or fat mass was observed (Massiera *et al.*, 2003). This elegantly highlights the critical role of omega-6 fatty acids and the PGI_2 pathway in the accumulation of fat. More recently Massiera *et al.* (2010) demonstrated that continually feeding a diet high in LA (19%en) resulted in a progressive increase in body fat mass over four successive generations, and this occurred in the absence of any differences in SFA intake between

groups. This increased fat mass was as a result of increased adipocyte hypertrophy and hyperplasia through the up-regulation of genes associated with these mechanisms.

The importance of dietary influences during post-natal and lactation periods has also been demonstrated. In the guinea pig, often considered the best rodent model of human adipose tissue development, newborns that were fed a low LA:ALA (2:1) milk formula and pellets up until postnatal day 21 had a significantly lower fat mass in later life than those fed a high ratio (36:1) (Pouteau *et al.*, 2010). Importantly, the dietary intervention only lasted until day 21, after which they were fed the same diet. At day 21 there were no differences in fat mass between groups highlighting the importance of this early window in the progression of obesity in later life. Another study by Korotkova *et al.* (2002) investigated the effects of an omega-6/omega-3 balanced maternal diet compared to either an omega-3 enriched diet or an omega-6 enriched diet on suckling offspring. The fatty acid profile of the milk as well as adipose tissue of the offspring largely reflected maternal dietary intakes. The results of this study also demonstrated that increased maternal intake of omega-3 fatty acids led to decreased growth rate and reduced adipose tissue mass in offspring at 3 weeks of age. This study highlighted the importance of the lactation period and capacity for the diet to affect breast milk fatty acid composition and it has in fact been shown that human breast milk fatty acid composition has shifted in the last 60 years (Figure 1.4) to reflect increasing dietary LA intake (Ailhaud *et al.*, 2006).

However, some confusion still remains around the effect of dietary PUFA on offspring adiposity as a study by Muhlhausler *et al.* (2011a) showed that offspring of rats fed a high omega-3 diet during pregnancy and lactation showed an increased total percentage body fat in both male and females. Few studies have investigated this hypothesis and it is clear that disparity between the results may be attributable to differences in species and study design (Muhlhausler *et al.*, 2011a).

1.7.2.2 Human studies

Few studies have focussed on the direct effects of increasing maternal omega-6 PUFA on offspring health. Instead, the majority of randomised control trials have investigated the effects of increasing omega-3 intake. One study by Donahue *et al.* (2011) reported a positive correlation between the omega-6:omega-3 ratio of cord blood and indicators

of increased fat mass (subscapular skinfold thickness and triceps skinfold thickness) in children at 3 years of age. A systematic review revealed very few studies examined the effect of PUFA supplementation during pregnancy on offspring body composition and results were highly variable (Muhlhausler *et al.*, 2011a). A more recent study, however, investigated the effect of maternal DHA supplementation on body fat mass and BMI in children at 7 years of age and found no differences between control and intervention groups (Wood *et al.*, 2018). Another more recent article reporting results from the INFAT study investigating the role of dietary PUFA during pregnancy and lactation on offspring adiposity, found no evidence that LCPUFA in maternal blood, cord blood or breast milk is predictive of offspring adiposity in a 5-year follow-up (Meyer *et al.*, 2019). This study is ongoing and will provide longitudinal insights into the effect of PUFA on adiposity.

1.8 Objectives and hypotheses

Whilst studies have highlighted the possible effect of varying the PUFA composition of the maternal diet on offspring body composition, often the ratio of omega-6:omega-3 is not representative of current intakes in human populations. In studies reporting increased offspring adiposity in response to a maternal diet high in omega-6, some ratios have been as high as 216:1 (Korotkova *et al.*, 2002) which is considerably higher than current estimated intakes.

Therefore, the primary objective of this research was to determine the effect of altering the quantity and quality of fat within the maternal diet on offspring body composition and health outcomes. Specifically we aimed to identify the effect of a maternal dietary LA:ALA ratio, similar to that consumed in the typical Western diet, against a more 'ideal' ratio on offspring growth and body composition. We hypothesised that a maternal diet high in LA would lead to increased offspring adiposity through increased adipogenic and lipogenic capacity of tissues and markers of this would be apparent in early life. We also aimed to identify whether any beneficial or detrimental effects observed with these two fatty acid profiles were exacerbated by increasing the total dietary PUFA content.

Objective 1: To investigate the effects of increasing the LA:ALA ratio, as well as total fat content, in the diet of rats during the gestational period only, on maternal weight gain, placental and fetal growth and the expression of lipogenic and adipokine genes in maternal and fetal tissues.

Objective 2: To investigate the effects of feeding an increasing maternal dietary LA:ALA ratio, as well as total fat content, on growth and fat deposition of the offspring who are directly exposed to experimental diets during pregnancy and lactation.

Objective 3: To investigate the effects of feeding an increasing maternal dietary LA:ALA ratio, as well as total fat content, on offspring adiposity and markers of CVD risk in young adulthood. This study will focus specifically on the effects of the maternal diet on offspring health post-lactation, where offspring have been weaned onto a standardised diet, and hence, investigate the longer-term consequences of possible early life programming of metabolic disturbance.

2 MATERIALS AND METHODS

2.1 Animal Diets

Animal diets were prepared in house at the University of Nottingham Bio-Support Unit. Diets were designed to provide a LA:ALA ratio of 9:1 (high LA) or 1:1.5 (low LA) which was achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The amount of SFA and MUFA were comparable in all diets, achieved by the manipulation of coconut (SFA source) and macadamia (MUFA source) oils in the diets. The fatty acid composition of each oil, as well as oil blends, was analysed using gas chromatography (see section 2.3.1.4) to ensure the correct fatty acid ratios were achieved (Table 2.1). For each level of LA, diets were made to contain either 18% or 36% fat by weight. Thus, resulting in four experimental diets; high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat). The gross composition of each diet is shown in Table 2.2. Diets were analysed in-house for energy content, fatty acid composition and protein content (Table 2.3). Protein content of experimental feeds was analysed using a Thermo Scientific nitrogen (N)/protein analyser (Flash EA1112). Percentage nitrogen was directly measured and protein (%) was estimated under the assumption of a 16% N content of proteins. Mineral and vitamin mixes were also produced in house according to the American Institute of Nutrition AIN-76A formulation (American Institute of Nutrition, 1977), compositions of which are shown in Table 2.4 and Table 2.5 respectively. For general maintenance and for weanling animals (where applicable) animals were provided with the 2018 Teklad Global 18% protein rodent diet (chow). The manufacturer's description of the composition can be found in Appendix A. Similarly to the experimental diets, an in-house analysis of fatty acid profile was also carried out for the chow diet, the results of which can be found in Appendix B.

Table 2.1 Fatty acid composition of oils and blends used in experimental diet

Fatty Acid	% of total fatty acids					
	Flaxseed Oil	Sunflower Oil	Macadamia Oil	Coconut Oil	9:1 (LA:ALA) Oil Blend	1:1.5 (LA:ALA) Oil Blend
8:0	-	-	-	5.33	1.01	0.94
10:0	-	-	-	5.17	0.99	0.91
12:0	-	-	-	48.02	9.26	8.39
14:0	0.04	0.08	0.47	20.38	4.09	3.72
16:0	5.27	6.89	11.48	10.13	9.43	9.63
17:0	0.07	0.04	0.04	0.01	0.04	0.04
18:0	3.64	3.28	3.18	3.06	3.27	3.30
20:0	0.44	0.25	1.31	0.09	0.65	0.82
22:0	0.11	0.74	0.68	0.02	0.56	0.44
24:0	0.09	0.26	0.28	0.04	0.22	0.20
Total SFA	9.66	11.54	17.44	92.27	29.52	28.38
18:1n-9	16.83	28.40	63.57	6.10	38.28	41.19
20:1n-9	0.13	0.17	1.12	0.04	0.54	0.66
22:1n-9	0.00	0.00	0.10	0.00	0.05	0.06
16:1n-7	0.04	0.12	8.66	0.01	3.52	4.72
18:1n-7	0.74	1.01	2.63	0.12	1.54	1.88
Total MUFA	17.74	29.70	76.09	6.28	43.92	48.50
18:3n-3	58.18	0.18	0.11	0.00	2.56	13.47
18:2n-6	14.31	58.50	6.24	1.43	23.94	9.59
Total PUFA	72.48	58.69	6.36	1.43	26.50	23.05

LA, linoleic acid; ALA, alpha linolenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2.2 Composition of experimental diets

Component	**Amount (g/100g diet)**			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
Casein	16.0	16.0	16.0	16.0
Cornflour	31.8	20.0	31.8	20.0
Sucrose	16.0	10.0	16.0	10.0
Cellulose	15.6	15.6	15.6	15.6
Mineral Mix[a]	1.8	1.8	1.8	1.8
Vitamin Mix[a]	0.4	0.4	0.4	0.4
Choline Chloride	0.2	0.2	0.2	0.2
Methionine	0.4	0.4	0.4	0.4
Flaxseed Oil	0.9	1.7	4.2	8.5
Sunflower Oil	6.6	13.1	0.8	1.7
Macadamia Oil	6.9	13.8	9.5	19.0
Coconut Oil	3.4	6.9	3.2	6.3
Total Fat	17.8	35.6	17.8	35.5

[a]Vitamin and mineral mixes were prepared according to the American Institute of Nutrition AIN-76A formulation (American Institute of Nutrition, 1977). LA, linoleic acid.

Table 2.3 Macronutrient content and major fatty acid composition of experimental diets

	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Macronutrients[a]				
Carbohydrates (% Energy)	46.0	23.8	46.0	23.8
Protein (% Energy)	15.4	12.7	15.4	12.7
Fat (% Energy)	38.6	63.5	38.6	63.5
Energy (MJ/kg)	21.2	24.5	21.3	24.8
Fatty Acids[b]				
Total SFA	20.7	20.3	21.1	20.0
Total TFA	0.1	0.1	0.1	0.1
Total MUFA	48.8	49.0	53.3	54.9
Total Omega-6 (LA only)	27.3	27.4	11.1	10.5
Total Omega-3 (ALA only)	3.1	3.2	14.4	14.4
Total PUFA	30.4	30.6	25.5	25.0
ALA (%Energy)	1.2	2.0	5.6	9.1
LA (%Energy)	10.5	17.3	4.3	6.7
LA:ALA Ratio	9.0	9.0	0.75	0.75

[a]Macronutrient %energy was calculated using Atwater factors

[b]Data represent fatty acid percentage of total fatty acids.

LA, linoleic acid; ALA, alpha linolenic acid; SFA, saturated fatty acids; TFA, trans fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2.4 Composition of mineral mix (AIN-76) used in rodent experimental diets

Mineral	Weight (g/kg)
Calcium phosphate dibasic	500.00
Sodium chloride	74.00
Potassium citrate	220.00
Sucrose	118.03
Potassium sulphate	52.00
Magnesium oxide	24.00
Ferric citrate	6.00
Manganese chloride	3.50
Zinc carbonate	1.60
Chromium potassium sulphate	0.55
Copper carbonate	0.30
Potassium iodate	0.01
Sodium selenite	0.01

Table 2.5 Composition of vitamin mix (AIN-76) used in rodent experimental diets

Vitamin	Weight (g/kg)
Thiamine hydrochloride	0.60
Riboflavin	0.60
Pyridoxine hydrochloride	0.70
Niacin	3.00
Calcium pantothenate	1.60
Folic acid	0.20
Biotin	0.02
Vitamin B12 (0.1%)	1.00
Vitamin A (500000IU/g)	0.80
Vitamin D3 (400000IU/g)	0.25
Vitamin E acetate (500IU/g)	10.0
Menadione neon bisulfite	0.08
Sucrose	981.15

2.2 Animal Trial Designs

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Virgin female Wistar rats (75-100g) were obtained from Charles River Laboratories (UK) and were housed in plastic individually ventilated cages (IVC) and subjected to a 12 h light–12 h dark cycle at a temperature of 20–22°C and 45-65% humidity. The animals were housed on wood shavings and had *ad libitum* access to food and water at all times. All experiments were performed within the University of Nottingham Bio-Support Unit (Sutton Bonington Campus, UK).

Animals were allowed to acclimatise to the unit for 2 weeks after arrival; during this time they were maintained on a standard laboratory chow diet (2018 Teklad Global 18% protein rodent diet). After two weeks, a tail vein blood sample was taken from rat dams using a needle to pierce the skin. This was done 10 minutes after the application of an anaesthetic cream. The initial blood drop was discarded and further drops were placed onto dried blood spot (DBS) cards (see section 2.3.1.1) for further analysis. Animals were then randomly allocated to their experimental diets and maintained on these for 4 weeks, during which time animals were pair-housed. A second tail vein blood sample was taken before animals were mated. After mating, animals followed the design of either trial I (see section 2.2.1) or trial II (see section 2.2.2). The two trials were run in parallel and four unrelated males were used for mating.

2.2.1 Trial I: Altered maternal diet and fetal outcomes

Trial I aimed to assess the effect of altered maternal fatty acid ratio and total fat content on fetal development.

2.2.1.1 Animal breeding and maintenance

Conception was confirmed by the appearance of a semen plug and this was noted as day 0 of pregnancy. Average time in mating pairs was 2.5 days and all but one female conceived within their first oestrous cycle housed with the male. After conception, animals were individually housed and remained on their respective diets until day 20 of gestation (full term = 22 days) where dams and fetuses were culled and tissues were

collected (see section 2.2.1.2). A schematic summary of the trial design can be seen in Figure 2.1. Dams were weighed daily, had feed intake measured and protein intake was estimated based on the protein content of the experimental diets (Table 2.3).

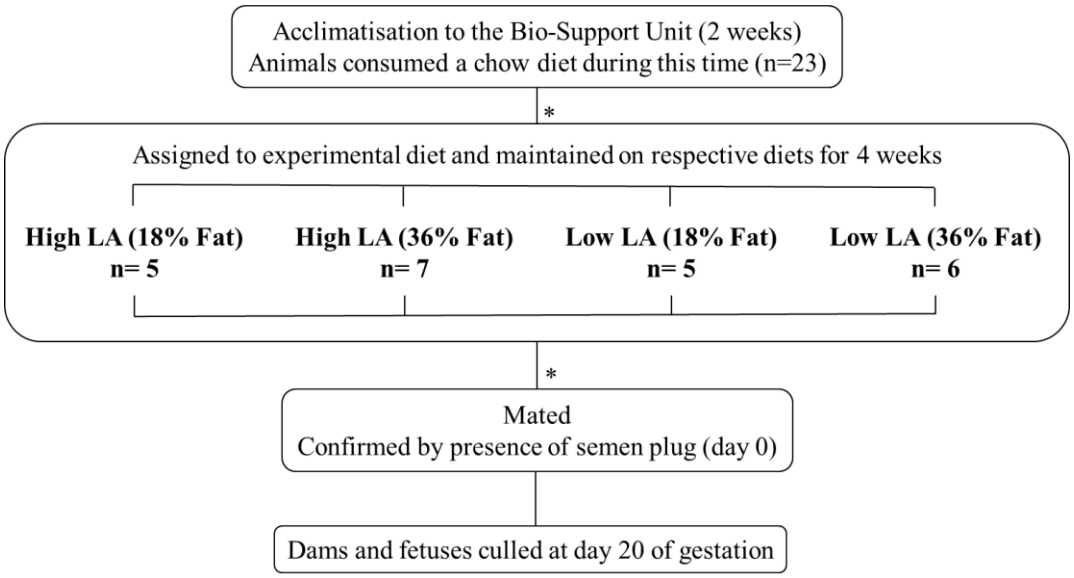


Figure 2.1 Schematic summary of animal trial I experimental design. *indicates collection of a tail vein blood sample for measurement of circulating fatty acids.

2.2.1.2 Cull and tissue collection

Animals were culled according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. Briefly, adult rats (>500g) were culled in rising carbon dioxide and death was confirmed by cervical dislocation. Fetuses were removed from the uterine horn, separated from the placenta and weighed, sexed via measurement of anogenital distance and then culled via decapitation; death was confirmed by exsanguination. Truncal blood samples were collected from two randomly selected male and two randomly selected female fetuses from each litter at this time. The whole blood samples (~30µl) were spotted onto DBS collection paper, allowed to dry at room temperature and were then stored at −20°C for subsequent fatty acid analysis. Fetal liver and placentas were weighed and samples of selected male placental and liver samples were collected. All tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Male fetuses selected for collection of tissue samples also had tail samples collected for sex-genotyping by PCR for the SRY gene (McClive and Sinclair, 2001). Any samples found to be female or inconclusive (n = 5) were not included in further analyses. Maternal tissues were weighed and samples of liver and

retroperitoneal adipose tissue were collected. Blood was collected from dams via cardiac puncture. Whole blood (~30µl) was spotted onto DBS cards and the rest was placed into heparinised tubes (Sarstedt) and centrifuged at 13000 rpm for 10 minutes at 4°C. The plasma was isolated from the whole blood samples and stored at -80°C until further analysis. All harvested organs were weighed and snap frozen in liquid nitrogen prior to storage at -80°C.

2.2.2 Trial II: Altered maternal diet and offspring outcomes up to 8 weeks of age

Trial II aimed to assess the effect of altered maternal fatty acid ratio and total fat content on offspring development up to 8 weeks of age.

2.2.2.1 Animal breeding and maintenance

Conception was confirmed by the appearance of a semen plug and this was noted as day 0 of pregnancy. Average time in mating pairs was 2.8 days and all but two females conceived within their first oestrous cycle housed with the male. After conception, animals were individually housed and remained on their respective diets until completion of the experiment. Litters were standardised to 8 pups within 24 hours of birth (4 males and 4 females where possible). At 1 and 2 weeks of age, one randomly selected male and one randomly selected female from each litter was culled. At 3 weeks of age, offspring were weaned away from the mother onto a chow diet. Animals were group housed with littermates according to sex. After the offspring were weaned the dams were culled. All remaining animals had blood pressure measurements taken at 4 weeks of age, after which one male and one female were randomly chosen and culled. At 8 weeks of age, all remaining offspring had another blood pressure measurement taken and were then culled. A schematic summary of the trial design can be seen in Figure 2.2. Dams were weighed daily, had feed intake measured and protein intake was estimated based on the protein content of the experimental diets (Table 2.3). All neonates were weighed at birth and sex was determined by measurement of anogenital distance. Offspring were then weighed weekly until the end of the experiment.

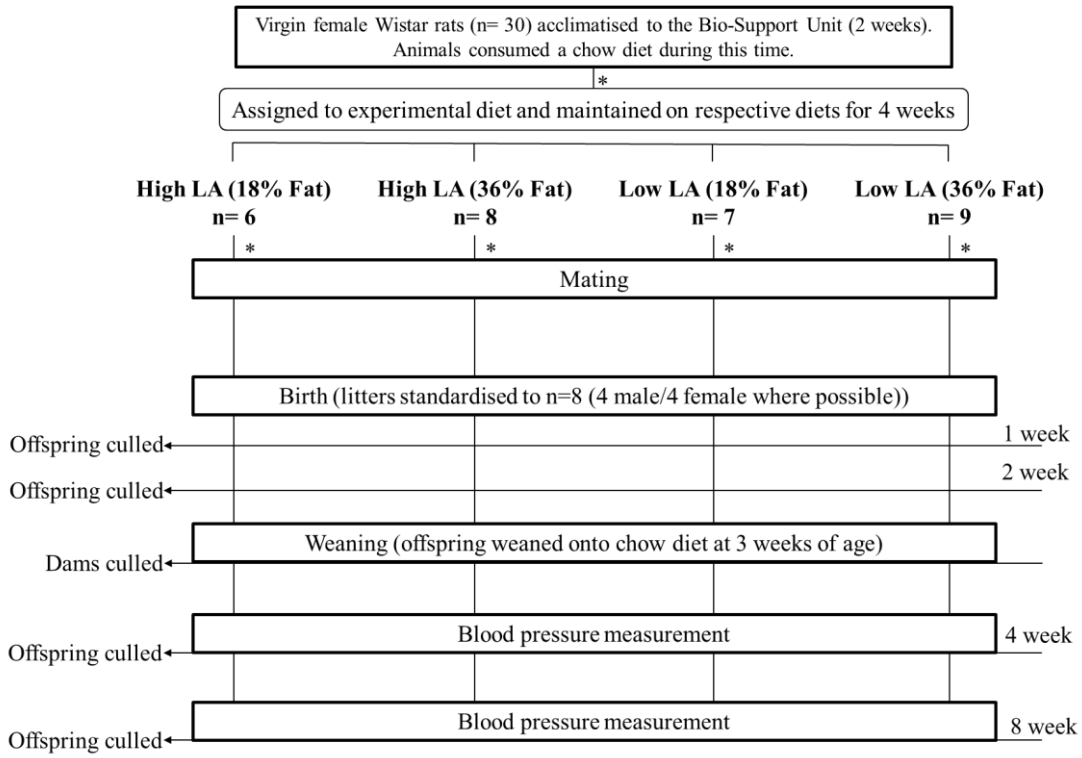


Figure 2.2 Schematic summary of animal trial II experimental design. *indicates collection of a tail vein blood sample for measurement of circulating fatty acids. Where offspring were culled (at 1, 2, 4 and 8 weeks of age), one male and one female were chosen at random from each litter.

2.2.2.2 Blood pressure measurements

This experiment relied on the use of a non-invasive, non-surgical method for the measurement of blood pressure. Non-invasive measurements of laboratory animals are usually taken in one of three ways: all involve the use of an occlusion cuff placed on the tail alongside either a LED sensor (photoplethysmography), a piezoelectric sensor (piezoplethysmography) or a volume pressure recording (VPR) sensor. This experiment utilised a VPR sensor which measured tail blood volume to deduce systolic, diastolic and mean arterial blood pressure as well as heart rate. This method was validated by Feng *et al.* (2008) against surgical telemetry methods, which are often considered the gold-standard for blood pressure measurements in laboratory animals. Tail-cuff measurements provided accurate measurements of blood pressure when compared with telemetry and only underestimated blood pressure by 0.25mmHg on average.

Prior to blood pressure measurements, animals were placed in a heat box set to 30°C for 15 minutes to enhance blood flow to the tail. Animals were then restrained in individual restraint tubes with adjustable nose cone, fitted with the deflated occlusion and VPR cuff (CODA System, Kent Scientific, Torrington, CT) and left to acclimatise to the restraint tube for 10 minutes to minimise the impact of stress before measurements began. After acclimatisation, animals underwent 10 cycles of blood pressure measurements. Of these 10 cycles, the first three were disregarded as acclimatisation cycles and an average for each measurement was taken from the remaining seven. Animals were restrained for no longer than 30 minutes and removed if individuals presented with signs of stress.

2.2.2.3 Cull and tissue collection

Animals were culled according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. Briefly, adult rats (>500g) were culled in rising carbon dioxide and death was confirmed by cervical dislocation. Adolescent rats (<500g) were culled via cervical dislocation and death was confirmed by exsanguination. Truncal blood was collected from offspring using glass capillary tubes and spotted onto DBS cards (~30µl) at 1 and 2 weeks of age. Blood was collected from dams and offspring at 4 and 8 weeks of age via cardiac puncture. Whole blood (~30µl) was spotted onto DBS cards and the remaining sample was placed in heparinised tubes (Sarstedt) and centrifuged at 13000 rpm for 10 minutes at 4°C. The plasma was isolated from the whole blood samples and stored at -80°C until further analysis. All harvested organs were weighed and snap frozen in liquid nitrogen prior to storage at -80°C.

2.3 Laboratory Methods

2.3.1 Lipid analysis

2.3.1.1 The use and validation of dried blood spot (DBS) cards for the measurement of fatty acids

The DBS technique is a sampling method where blood is spotted onto filter paper and allowed to dry. This drying stabilises the blood sample for further analysis and the technique has been in use since the 1960s where it was first used to screen for phenylketonuria in infants (Guthrie and Susi, 1963). Since then it has been used as a

screening method for biomarkers for a range of disorders such as HIV (Parker and Cubitt, 1999), cystic fibrosis (Wilcken *et al.*, 1983) and sickle-cell anaemia (Garrick *et al.*, 1973). Advances in the sensitivity and specificity of techniques associated with analysing small volumes of blood have resulted in the DBS technique becoming a significant tool, particularly in newborn screening.

When compared with conventional blood collection and measurement techniques, the DBS technique has many advantages (Parker and Cubitt, 1999). One such advantage is that the DBS technique only requires the collection of capillary blood, a significant benefit especially in neonates and elderly patients where venous blood collection can be very difficult. The technique also eliminates the need for immediate processing of blood samples to separate blood fractions for long-term stability and storage. Because of the immediate stability to samples that the DBS technique provides, blood samples can be collected 'in field' and shipped, at room temperature, for analysis at a later time.

Most of the advantages that the DBS technique confers over traditional venepuncture methods centre on the immediate stability of the blood samples, more specifically, the stability of the metabolite of interest within the blood sample. The use of the DBS technique to measure fatty acid status was first described by Nishio *et al.* (1986) but required a relatively large volume (~100µl) of blood and was validated for specific fatty acid biomarkers of adrenoleukodystrophy which are inherently stable in air (C22:0, C24:0 and C26:0). Since then, improvements have been made in the technique, however, the inability to measure fatty acids in all lipid fractions (Ichihar *et al.*, 2002) and significant variability in measurements of LCPUFA when compared to conventional techniques (Marangoni *et al.*, 2004), limited the application of this method for the measurement of the complete fatty acid profile. The oxidation of fatty acids, particularly LCPUFA, when exposed to air was postulated as a potential reason for this high variability (Ichihar *et al.*, 2002). As such, to stabilise the LCPUFA, studies took the approach of either processing the samples immediately (Agostoni *et al.*, 2008) or storing them at -20°C (Bell *et al.*, 2011), both of which negate the advantage of the method as a convenient and stable technique.

Previous research in our laboratory by Liu *et al.* (2014) established and validated a method for the measurement of the complete fatty acid profile in whole blood (PUFA coat™). This work tested fatty acid stability on various commercially available filter

papers all of which exhibited significant oxidation of LCPUFA. Coating the paper in silica gel increased fatty acid binding and the treatment of filter paper with an antioxidant and a chelating agent significantly reduced the oxidation effect of LCPUFA observed in previous studies. The stability of fatty acids was established for storage at room temperature for up to 2 months. Liu *et al.* (2014) validated the method in 50 human subjects and found strong correlations of all fatty acids when compared to conventional techniques. As well as the accurate determination of total lipid content within a sample, the amount of separate lipid classes can also be determined including phospholipids, free fatty acids, triglycerides and cholesterol esters.

This method was used for the analysis of fatty acids in blood samples in this study due to its convenience over conventional techniques. Further to this, it allowed for the measurement of small volumes of blood which was critical for determining fetal and early postnatal fatty acid profiles where sufficient amounts of blood could not be collected for conventional processing techniques (Figure 2.3). It also minimised pain and distress where blood samples were taken from live animals. Blood samples were spotted onto PUFA-coat™ DBS collection cards (FOODplus Research Centre, University of Adelaide) and stored at 4°C for a maximum of 2 weeks before postage to the Waite Lipid Analysis Service, University of Adelaide, where samples were stored at -80°C until further analysis.

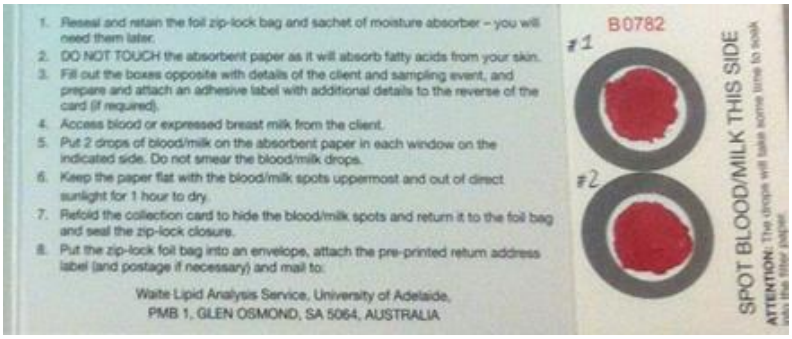


Figure 2.3 Example of dried blood spot (DBS) sample obtained from newborn rat offspring on a PUFA-coat™ DBS collection card (Liu *et al.*, 2014).

2.3.1.2 Fatty acid extraction from DBS cards and synthesis of fatty acid methyl esters (FAME)

Fatty acid composition of DBS samples was determined as described by Liu *et al.* (2014). DBS samples were cut from the protective card (Figure 2.3) and directly transesterified with 2ml of 1%H_2SO_4 in methanol at 70°C for 3 hours. Samples were vortexed every 30 minutes during this time. After cooling, 250µl of distilled water and 0.6ml of heptane were added to each sample. Samples were vortexed and then allowed to stand for 10 minutes to allow for separation of layers. The top heptane layer containing FAME was pipetted into a fresh glass vial containing anhydrous sodium sulphate (Na_2SO_4) as a desiccant. Samples were then analysed using gas chromatography (see section 2.3.1.4)

2.3.1.3 Fatty acid extraction from tissues and FAME synthesis

Total lipids were extracted from placental samples in chloroform/isopropanol (2:1 v/v) using an adaptation of the Folch method (Folch *et al.*, 1957). Crushed, frozen placenta was weighed (~300mg) and added to 2ml of 0.9% saline solution. The solution was homogenised on ice before the addition of 3ml isopropanol. The solution was vortexed and left to stand for 5 minutes. After this, 6ml of chloroform was added and the solution was vortexed. The samples were then centrifuged at 3000 rpm for 15 minutes and the bottom chloroform layer containing the extracted lipid was transferred into a fresh tube. This was then dried down under nitrogen before being reconstituted in chloroform/methanol (9:1 v/v). For logistical reasons 20µl of the sample was then spotted onto PUFA-coat™ DBS collection cards and FAME synthesis was carried out as per section 2.3.1.2.

Total lipids were extracted from liver samples of 4 and 8-week-old offspring. For each sample, ~300mg of crushed, frozen liver was homogenised in 1.6ml of 0.5M Na_2SO_4. The homogenate was decanted into 5.4ml of hexane-isopropanol (3:2, v/v) and 2ml of 0.5M Na_2SO_4 was added. Samples were vortexed and then centrifuged at 3000 rpm for 15 minutes. The supernatant was removed into a fresh tube, dried under nitrogen and the resultant lipid content was weighed. Samples were resuspended in 1ml of hexane and 100µl of resuspended sample was removed into a fresh tube, re-dried under nitrogen and resuspended in 100µl of isopropanol for the determination of cholesterol

and triglyceride content (see section 2.3.3.4 and 2.3.3.5). The remaining sample was stored at -20°C for fatty acid analysis (see section 2.3.1.4).

2.3.1.4 Gas chromatography (GC) analysis of fatty acid methyl esters (FAME)

Samples containing FAME were analysed at the University of Adelaide. Samples were separated and analysed by a Hewlett-Packard 6890 gas chromatograph (GC) equipped with a capillary column (30m x 0.25mm) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70; 0.25µm film thickness) which was fitted with a flame ionisation detector (FID). Helium was utilised as the carrier gas and a split ratio was set at 20:1. Injector temperature was set at 250°C and FID temperature at 300°C. FAME were identified in unknown samples based on the comparison of retention times with an external lipid standard (Standard 463, Nu-check prep Inc. MN, USA) using Agilent Chemstation software. Individual fatty acid content was calculated based on peak area and response factors normalised to total fatty acid content and expressed as a percentage of total fatty acids across lipid classes.

2.3.2 Gene expression analysis

2.3.2.1 Isolation and preparation of ribonucleic acid (RNA) from liver

RNA was isolated from liver using Roche High Pure Tissue kits (Roche Diagnostics Ltd, UK). Lysis buffer (400µl) was added to ~25mg of crushed snap-frozen liver tissue in a clean, autoclaved Eppendorf tube. Samples were further homogenised by passing the tissue and buffer through a 21-gauge needle 5-10 times. The lysate was then centrifuged for 2 minutes at 13000 rpm and the supernatant was removed and placed into a fresh sterile Eppendorf tube with 200µl of absolute ethanol. A Roche high pure filter tube was combined with the collection tube and the entire solution was pipetted into the upper chamber of the filter/collection assembly. The samples were then centrifuged for 30 seconds at 13000 rpm and the filtered flow-through was discarded. For each sample, 90µl of DNase incubation buffer was mixed with 10µl DNase and the solution was added to the upper chamber of the filter/collection assembly in order to remove any contaminating genomic DNA. After an incubation step of 15 minutes at room temperature, the assembly was sequentially washed with wash buffers I and II. The upper chamber was then removed and placed in a fresh sterile Eppendorf tube

and 100µl of elution buffer was passed through (centrifuged at 8000 rpm) and the resultant solution in the Eppendorf contained the extracted RNA.

2.3.2.2 Isolation and preparation of RNA from adipose tissue

RNA was isolated from adipose tissue samples using a QIAGEN RNeasy Mini-kit (QIAGEN Ltd., UK) alongside MagNA Lyser green beads and instrument (Roche Diagnostics Ltd.). Lysis buffer (600µl; containing 1% beta-mercaptoethanol) was added to ~100mg of crushed snap frozen adipose tissue in a sterile tube containing the MagNA Lyser green beads. Samples were vortexed for 10 seconds and placed in the MagNA Lyser instrument for 40 seconds at 6500 rpm. Samples were then centrifuged at 8000 rpm for 3 minutes. After centrifugation, 400µl of the infranatant was removed (carefully avoiding the top lipid layer) into a fresh sterile Eppendorf tube and kept on ice. To increase RNA yield, the previous steps were repeated after adding 350µl of lysis buffer to the remaining solution in the tube containing the MagNA Lyser green beads. The resulting infranatant was then combined with that collected previously. The sample was then centrifuged for 5 minutes at 13000 rpm and 700µl of the supernatant removed and added to an equal volume of 70% ethanol. The resulting solution was then incrementally passed through the RNeasy filter column (centrifuged at 800 rpm for 15 seconds) and flow-through discarded. After a wash step, 70µl of buffer was combined with 10µl of DNase and pipetted into the top chamber of the RNeasy column where it was incubated for 15 minutes at room temperature. After multiple subsequent wash steps, the upper chamber of the RNeasy column was placed in a fresh sterile Eppendorf tube and 30µl of RNase-free water was added. The assembly was centrifuged for 1 minute at 8000 rpm and the resulting solution in the Eppendorf contained the eluted RNA.

2.3.2.3 Testing RNA concentration and purity

All RNA samples were tested for RNA concentration (ng/µl) using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Samples were measured at 260nm for nucleic acids and at 280nm for proteins. The 260/280 ratio gave an indication of any protein contamination within the samples and those failing to achieve a ratio value between 1.8 and 2.0 and/or concentration lower than 50ng/µl were discarded and RNA extraction was repeated as in section 2.3.2.1 or 2.3.2.2.

2.3.2.4 Agarose gel electrophoresis

RNA quality was assessed by agarose gel electrophoresis. All samples were diluted to a concentration of 50 ng/µl with RNAse free water based on the concentrations measured in section 2.3.2.3. A 1% agarose gel was prepared by diluting 1.5g agarose in 150ml 1xTris-acetate-EDTA (TAE) buffer. The solution was microwaved until the agarose was completely dissolved, cooled briefly under running water, and immediately poured into a gel tray with a 20 well comb inserted. Gels were left to set for 45 minutes after which the comb was removed and the gel was placed in the gel tank and submerged with 1xTAE buffer. Sample was loaded (10µl) alongside 2µl of loading dye into each well. The gel was electrophoresed at 100v for approximately 30 minutes or until the bands were ~2cm from the bottom of the gel. The gel was then stained in ethidium bromide for 20 minutes and imaged under UV light (Gel Doc 2000, Bio-Rad Laboratories Inc.). After visual inspection of the gel, samples with two clear bands were considered acceptable. Any samples presenting with bands that were unclear, absent or smeared were discarded and RNA extraction was repeated as in section 2.3.2.1 or 2.3.2.2.

2.3.2.5 Complementary DNA (cDNA) synthesis

Qualifying RNA samples were synthesised into first strand complementary DNA (cDNA) using RevertAid™ Reverse Transcription kit (Thermo Fisher Scientific Inc.). Reaction mixes were prepared according to manufacturer's instructions. A master mix containing random hexamer primers and nuclease free water was added to RNA into a fresh 96 well plate (Table 2.6) and incubated at 65°C for 5 minutes (GeneAmp PCR system 9700, Applied Biosystems). A 'no template control' was also added to each plate where RNA was replaced with water to ensure there was no contamination of the reagents. The plate was then immediately placed on ice to halt any further reactions. A second master mix was prepared (Table 2.6) and 8µl was added to each well and the plate was then incubated once more for 5 minutes at 25°C followed by 60 minutes at 42°C. The reaction was terminated by heating the plate to 70°C for 5 minutes. A further 80µl of RNase free water was added to each sample to make each well up to a 100µl total volume. A further dilution of the cDNA (1:8) was made and the plate was stored at -20°C until further analysis.

Table 2.6 Components of the master mixes prepared for the RevertAid™ reverse transcription reaction

Component	Volume
Master mix I	
Total RNA (50ng/ml)	10µl
Random hexamer primer	1µl
RNase free water	1µl
Master mix II	
5x Reaction buffer	4µl
RiboLock RNase inhibitor	1µl
10mM dNTP mix	2µl
RevertAid™ RT	1µl

2.3.2.6 Designing of primer pairs and primer sequences

Oligonucleotide primers were designed for each experiment using online databases and Primer Express software, with the exception of *Fads1* and *Fads2* primers which were purchased from Qiagen (*Fads1*; Rn_Fads1_1_SG QuantiTect Primer Assay, Qiagen; *Fads2*; Rn_Fads2_1_SG QuantiTect Primer Assay, Qiagen). The gene sequence was selected in NCBI (National Centre for Biotechnology Information), and the exon-intron boundaries identified using Ensembl Genome Browser. This sequence information was inserted into Primer Express in FASTA format (Software Version 3.0; Applied Biosystems) and primers generated that cross an exon-intron junction to increase specificity. Newly designed primer pairs were checked using an online Basic Local Alignment Search Tool (BLAST) for alignment with the sequence of interest and to ensure no other sequences were detected by the primers. Selected primers were ordered via Sigma-Aldrich Custom Oligos. Primer sequences can be found in relevant results chapters.

2.3.2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

Although the basic principle is similar, the polymerase chain reaction (PCR) can be performed in a variety of ways to measure gene expression in various biological samples. Single stranded cDNA is heated with a specific primer pair, designed to bind and amplify a target sequence i.e. the gene of interest. This amplification occurs over a number of amplification cycles. During each cycle, each single stranded cDNA sample is denatured by increasing the temperature. The temperature is then lowered again to allow for primer annealing. Finally the temperature is raised again to allow a thermally stable DNA polymerase to amplify the target sequence. The quantity of amplification is relative to the amount of starting sequence within the sample giving an indication of the overall abundance of the gene of interest.

This research relied on a specific method for the measurement of gene expression, quantitative real-time PCR (qRT-PCR). This method utilises a fluorescent dye which binds to double stranded nucleic acids. As PCR progresses, the fluorescent signal will increase. With each cycle, the number of copies of the gene of interest doubles and so in theory the fluorescent signal should double also. At the start of the reaction however, as there are only a few template strands, the signal is not strong enough to be detected (lag phase). After a few more cycles the gene of interested is amplified in a higher quantity and the fluorescent signal can be detected above the background noise (exponential phase). After a few more cycles, the reaction will then begin to slow down as the reagents are used up (plateau phase). As such, it is important to ensure that quantification of expression and any analysis performed is based upon data generated during the exponential phase, measurements during the lag or plateau phase are not entirely representative of starting quantities as they are limited by reagent quantity. The cycle in the exponential phase where the fluorescent signal moves into the detectable range is called the threshold cycle (C_T). The amount of starting material within each sample will dictate how long it takes for the reaction to enter its C_T: a lower amount of starting material would require more cycles and so the C_T would be higher.

For this research, a ‘master mix’ containing SYBR green fluorescent dye (Roche Diagnostics), forward and reverse primers for the target gene and RNAse free water were made. Exactly 10µl of the master mix and 5µl of the cDNA sample were pipetted

into each well of a 384-well LightCycler® plate (Roche diagnostics) in triplicate. Plates were covered and set to run on a LightCycler®480 instrument (Roche diagnostics) with the programme outlined in Table 2.7.

Table 2.7 Phases of the qRT-PCR programme

Phase of reaction	Number of cycles	Settings per cycle
Pre-incubation	1	95°C (5 minutes)
Amplification	45	95°C (10 seconds) 60°C (15seconds) 72°C (15 seconds)
Melting curve	1	95°C (5 seconds) 60°C (1 second) 90°C (cont.)
Cooling	1	40°C

To ensure there was no contamination, each data-set of cDNA was analysed alongside their respective RNA samples. If any amplification was detected in the RNA during the PCR cycles then this RNA/cDNA sample was excluded. In order to obtain quantitative data, subsequent samples were run alongside a standard curve of serially diluted cDNA pool, ranging from neat to 1:128 dilution. The cDNA pool was made up by adding 10µl of neat cDNA from each sample to make a stock. The qRT-PCR experiments were normalised to housekeeper genes that were not affected by dietary treatment. Further details can be found within the respective results chapters.

2.3.3 Assays

2.3.3.1 Determination of liver DNA concentration

Motivated by differences in liver weight, DNA content of the liver was assessed in Chapter 4 to elucidate whether changes in tissue weight were attributable to differences in cell number (hyperplasia). The DNA concentration of liver samples was analysed using an adaptation of the Hoechst fluorometric assay method. Samples were crushed under liquid nitrogen and ~10mg of crushed tissue was added to 100µl of 0.05M trisodium citrate buffer. The solution was homogenised thoroughly and centrifuged at

2500 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was discarded.

Serial dilutions of the stock DNA (DNA from calf thymus (1mg/ml); Sigma-Aldrich, UK) were prepared to give standards ranging from 0.3125-20µg/ml. After this, 100µl of distilled water (blank), standard or sample (10µl homogenate in 90µl H_2O) was added to a 96-well plate. The dye solution (100µl of Hoechst 33258 dye (1mg/ml) in 15ml 2x TNE buffer) was added to each well and fluorescence was measured using a FLUOstar Omega microplate reader (BMG Labtech Ltd.) with an excitation wavelength of 355nm and emission wavelength of 460nm. Standards and samples were analysed in duplicate and the DNA concentration was quantified against the standard curve and normalised to tissue weight. The inter-assay and intra-assay coefficients of variation were 10.4% and 2.1% respectively. A composite standard curve is shown in Figure 2.4.

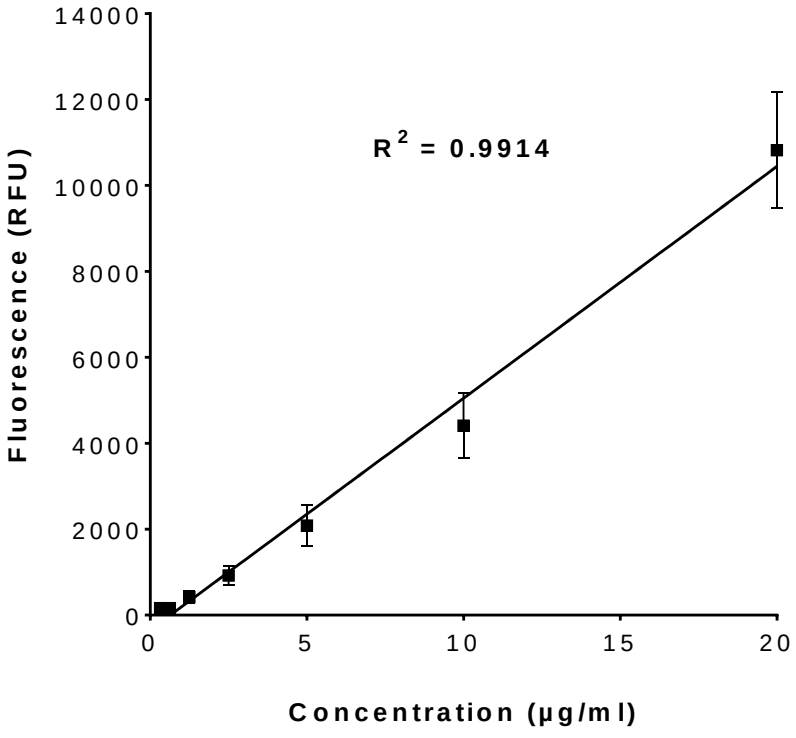


Figure 2.4 Composite standard curve from four DNA assays. Data presented are mean ± SD.

2.3.3.2 Determination of liver protein concentration

Motivated by differences in liver weight, protein content of the liver was assessed in Chapter 4 to elucidate whether changes in tissue weight were attributable to differences in cell size (hypertrophy). The concentration of liver protein was analysed as a marker of cell size. Liver protein samples were prepared as described in section 2.3.3.1 and protein concentration was determined using a colorimetric method as described by Lowry *et al.* (1951), modified for a 96 well plate format. Serial dilutions of bovine serum albumin stock (5mg/ml) were prepared to give standards ranging from 0.2-1.2mg/ml. Either 50µl of standard or sample (2µl homogenate in 48µl H_2O) was added to each well with 150µl of 0.1M NaOH and 50µl of freshly prepared solution 1 (Table 2.8). The plate was then incubated for 5 minutes at room temperature. After this, 50µl of freshly prepared solution 2 (Table 2.8) was added and samples were incubated again at room temperature for 20 minutes. The absorbance was measured at 655nm on a microplate reader (Bio-Rad 680-XR). Standards and samples were analysed in duplicate and the protein concentration was quantified against the standard curve and normalised to tissue weight. The inter-assay and intra-assay coefficients of variation were 11.7% and 2.5% respectively. A composite standard curve is shown in Figure 2.5.

Table 2.8 Working solutions for the quantification of protein in liver samples.

Component	Volume (ml)
Solution 1	
Na_2CO_3 (2%w/v) in 0.1M NaOH	5
$CuSO_4$ (1% w/v)	0.5
KNa Tartrate (2%w/v)	0.5
Solution 2	
0.1M NaOH	5
Folin–Ciocalteu reagent	0.5

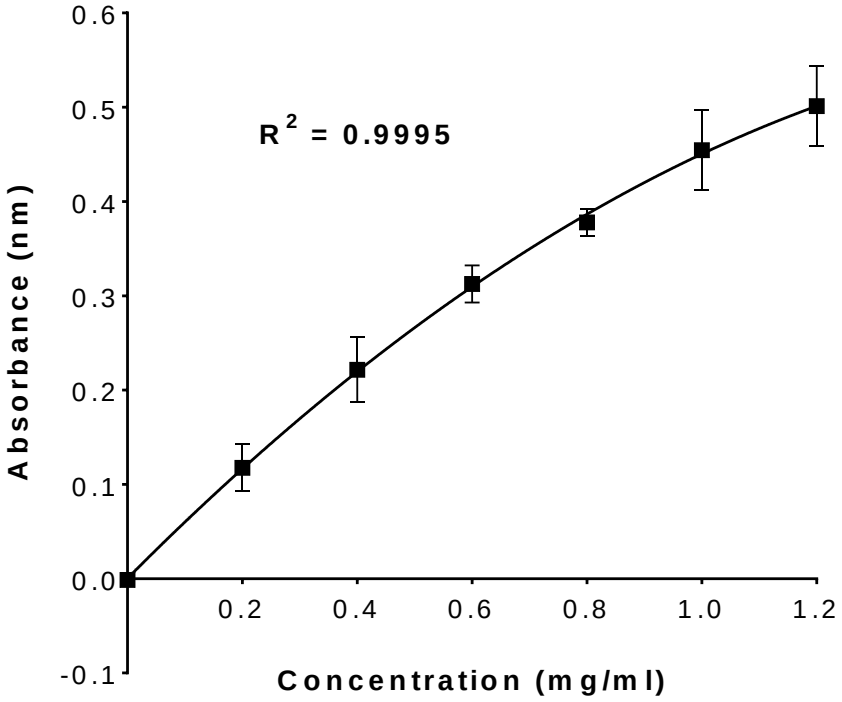


Figure 2.5 Composite standard curve from four protein assays. Data presented are mean ± SD.

2.3.3.3 Determination of liver glycogen concentration

Liver glycogen was measured using the Colorimetric Glycogen Assay Kit II (Abcam Ltd.) according to manufacturer's instructions. This assay relies on glycogen being hydrolysed to glucose, which is then oxidized to form an intermediate that reduces a colourless probe to a coloured product. As such, it is not possible to distinguish between the glucose derived from glycogen and any glucose that was originally in the sample. As such, for every sample a blank was also prepared alongside that lacked the enzyme required for the hydrolysis of glycogen to glucose.

Samples were crushed under liquid nitrogen and ~20mg of tissue was added to 400µl of distilled water. Samples were homogenised (60 seconds at 6500rpm) using a MagNA Lyser instrument and MagNA Lyser green beads (Roche Life Science) and then immediately boiled at 100°C for 10 minutes. Samples were then centrifuged at 13000rpm for 10 minutes at 4°C. The supernatant was collected (200µl) into a fresh tube.

Standards were prepared from a glycogen standard stock (2mg/ml). This stock was diluted to 0.2mg/ml in water and then 0-10µl of the stock was added to each well (made up to 50µl with glycogen hydrolysis buffer) to produce a standard curve ranging from 8-40µg/ml. The supernatant from homogenised tissue was diluted 1:10 and 10µl of this dilution was used for the assay to give approximately 50µg/well and wells were made up to 50µl with glycogen hydrolysis buffer. After this, 2µl of the hydrolysis enzyme mix was added to all standard and sample wells but was not added to sample blanks. The plate was then incubated at room temperature for 30 minutes.

During this time, a reaction mix was prepared for the standards and samples and a background control mix was prepared for the sample blanks (Table 2.9). To all standard and sample wells, 48µl of the reaction mix was added and 50µl of the background control mix was added to all sample blanks. The assay was incubated at room temperature for 30 minutes and then absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech Ltd.) at 450nm. Standards and samples were analysed in duplicate and the glycogen concentration was quantified against the standard curve and normalised to tissue weight. The inter-assay and intra-assay coefficients of variation were 2.8% and 8.7% respectively. A composite standard curve is shown in Figure 2.5.

Table 2.9 Glycogen assay reaction and background control mix working solutions

	Reaction mix (µl)	**Background control mix (µl)**
Glycogen development buffer	44	46
Development enzyme mix	2	2
Probe	2	2

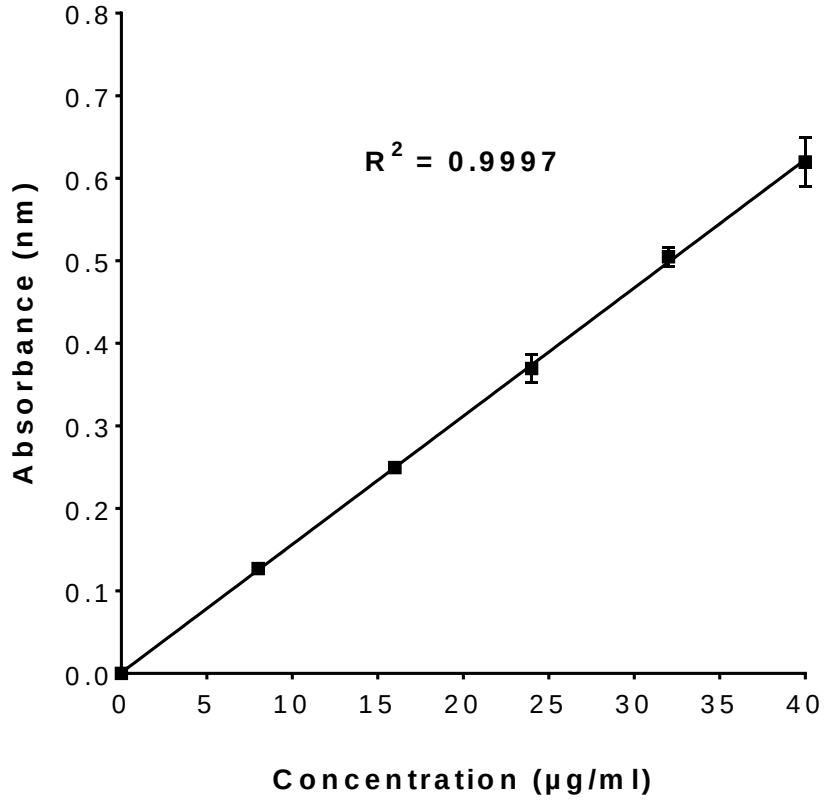


Figure 2.6 Composite standard curve from five glycogen assays. Data presented are mean ± SD.

2.3.3.4 Determination of plasma and liver cholesterol concentration

Total lipid was extracted from liver samples as described in section 2.3.1.3. Cholesterol concentration was determined using an Infinity™ cholesterol liquid stable reagent (Thermo Fisher Scientific) based on a formulation by Allain *et al.* (1974) later modified by Roeschlau *et al.* (1974). Serial dilutions of a 5.7mmol/L sCal calibrator solution (Thermo Fisher Scientific) were prepared to give a standard curve ranging from 0.36-5.7mmol/L. Exactly 10µl of standard or sample was pipetted onto a 96 well microplate and 250µl of Infinity™ cholesterol reagent was added. The plate was incubated for 5 minutes at 37°C and absorbance was read at 550nm in a microplate reader (Bio-Rad 680XR). Standards and samples were analysed in duplicate and the cholesterol concentration was quantified against the standard curve and liver cholesterol was normalised to tissue weight. The inter-assay and intra-assay coefficients of variation were 11.4% and 4.5% respectively. A composite standard curve is shown in Figure 2.7.

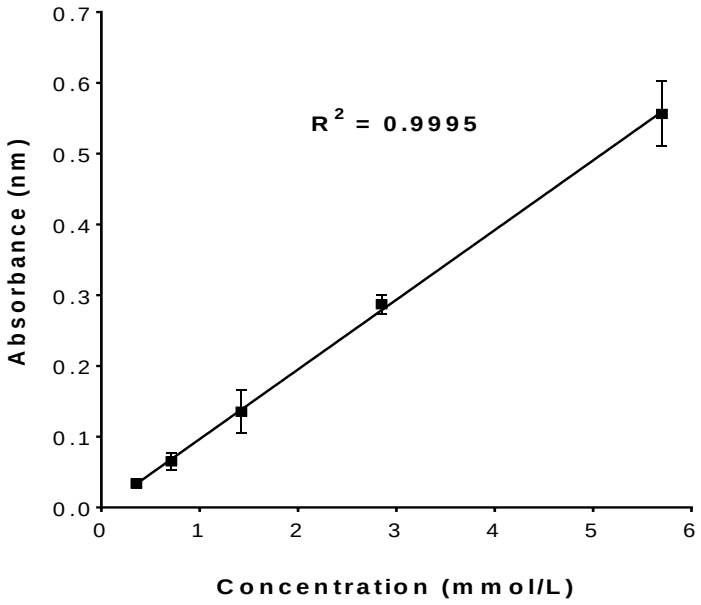


Figure 2.7 Composite standard curve from eight cholesterol assays. Data presented are mean ± SD.

2.3.3.5 Determination of plasma and liver triglyceride concentration

Total lipid was extracted from liver samples as described in section 2.3.1.3. Triglyceride concentration was determined using an Infinity™ triglyceride liquid stable reagent (Thermo Fisher Scientific) based on methods outlined by Fossati and Prencipe (1982) and McGowan *et al.* (1983). Serial dilutions of a 2.14mmol/L sCal calibrator solution (Thermo Fisher Scientific) were prepared to give a standard curve ranging from 0.13-2.14mmol/L. Exactly 10µl of standard or sample was pipetted onto a 96 well microplate and 250µl of Infinity™ triglyceride reagent was added. The plate was incubated for 5 minutes at 37°C and absorbance was read at 550nm in a microplate reader (Bio-Rad 680XR). Standards and samples were analysed in duplicate and the triglyceride concentration was quantified against the standard curve and liver triglyceride was normalised to tissue weight. The inter-assay and intra-assay coefficients of variation were 4.9% and 3.7% respectively. A composite standard curve is shown in Figure 2.8.

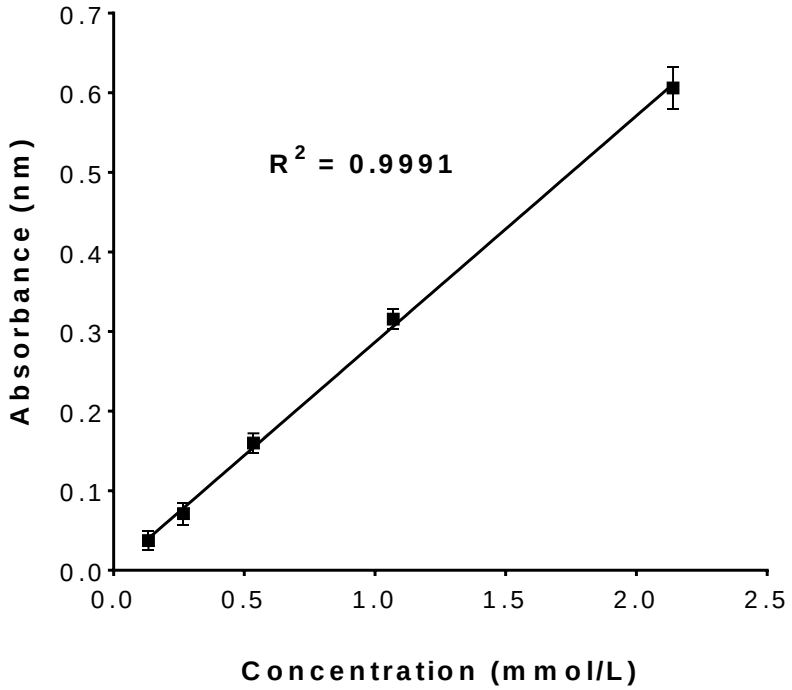


Figure 2.8 Composite standard curve from eight triglyceride assays. Data presented are mean ± SD.

3 MANUSCRIPT 1: THE ROLE OF MATERNAL DIETARY LA:ALA RATIO ON FETAL AND PLACENTAL GROWTH

Draycott, SAV, Liu, G, Daniel, ZC, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Maternal dietary ratio of linoleic acid to alpha-linolenic acid during pregnancy has sex-specific effects on placental and fetal weights in the rat. *Nutrition & Metabolism*, 16(1), 1.

Author contributions can be found in Appendix C and the published article can be found in Appendix D.

3.1 Abstract

Background: Increased consumption of linoleic acid (LA, omega-6) in Western diets coupled with the pro-inflammatory and adipogenic properties of its derivatives has led to suggestions that fetal exposure to this dietary pattern could be contributing to the intergenerational cycle of obesity.

Method: This study aimed to evaluate the effects of maternal consumption of a LA to alpha-linolenic acid (ALA) ratio similar to modern Western diets (9:1) compared to a lower ratio (1:1.5) on placental and fetal growth, and to determine any cumulative effects by feeding both diets at two total fat levels (18% vs 36% fat w/w). Female Wistar rats (n=5-7/group) were assigned to one of the four experimental diets prior to mating until 20d of gestation.

Results: Fatty acid profiles of maternal and fetal blood and placental tissue at 20d gestation were different between dietary groups, and largely reflected dietary fatty acid composition. Female fetuses were heavier (2.98±0.06g vs 3.36±0.07g, $P<0.01$) and male placental weight was increased (0.51±0.02g vs 0.58±0.02g, $P<0.05$) in the low LA:ALA groups. Female fetuses of dams exposed to a 36% fat diet had a reduced relative liver weight irrespective of LA:ALA ratio (7.61±0.22% vs 6.93±0.19%, $P<0.05$). These effects occurred in the absence of any effect of the dietary treatments on maternal bodyweight, fat deposition or expression of key lipogenic genes in maternal and fetal liver or maternal adipose tissue.

Conclusion: These findings suggest that both the total fat content as well as the LA:ALA ratio of the maternal diet have sex-specific implications for the growth of the developing fetus.

3.2 Introduction

The prevalence of obesity, a major risk factor for cardiovascular disease and type 2 diabetes, continues to rise in both low-, middle- and high-income countries, with 1.9 billion adults worldwide estimated to be overweight or obese as of 2016 (WHO, 2017a). Of particular concern is that 41 million children under the age of 5 were estimated to be overweight or obese globally in 2016 (WHO, 2017a), and often present with early onset of cardiometabolic diseases including type 2 diabetes and hypertension (Owen *et al.*, 2009, Mayer-Davis *et al.*, 2017).

Exposure to either an inappropriately high or inappropriately low plane of nutrition before birth and/or in early infancy is a major risk factor for the development of obesity and type 2 diabetes through the life course (Gale *et al.*, 2007, Catalano and Ehrenberg, 2006, Muhlhausler *et al.*, 2006, Langley-Evans, 2015). Maternal high-fat diets have consistently been associated with an increased risk of obesity and poor cardiometabolic health in the offspring (Armitage *et al.*, 2005). It is increasingly recognised, however, that the impact of maternal fat consumption is dependent not only on the amount of fat consumed but also the specific fat type. The ratio of omega-6 and omega-3 polyunsaturated fatty acids (PUFA) in the diet appears to be of particular importance, likely due to the opposing roles of these two classes of PUFA on metabolic and inflammatory processes within the body (Schmitz and Ecker, 2008). Thus, omega-3 PUFA, including alpha-linolenic acid (ALA) and its long chain PUFA (LCPUFA) derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibit inflammation, adipogenesis and lipogenesis (Massiera *et al.*, 2003, Madsen *et al.*, 2005). Conversely, the omega-6 PUFA, linoleic acid (LA) and its long chain derivative, arachidonic acid (AA) have pro-inflammatory actions and promote adipogenesis and lipogenesis *in vitro* and *in vivo* (Shillabeer *et al.*, 1998, Ailhaud *et al.*, 2006). Consequently, excess consumption of omega-6 relative to omega-3 fatty acids would be expected to be associated with increased incidence of inflammatory conditions, increased adiposity and heightened risk of cardiometabolic diseases.

The opposing roles of the omega-3 and omega-6 fatty acids have particular relevance due to a substantial increase in the intake of the omega-6 essential fatty acid, LA, in the Western diet over the past few decades, with very little change in the intake of omega-3 PUFA. The ratio of LA:ALA in typical Western diets has been reported at

~8-10:1 (Blasbalg *et al.*, 2011, Meyer *et al.*, 2003) or higher (Simopoulos, 2016), which is substantially higher than the proposed 'ideal' ratio of ~1-2:1 based on levels required to achieve maximal conversion of ALA to its longer chain derivatives (Lands, 2008, Tu *et al.*, 2010, Gibson *et al.*, 2011). Previous studies have shown that the high omega-6:omega-3 ratio in the modern Western diets is reflected in the blood and tissue fatty acid profiles of pregnant and lactating women, as well as breast milk (Ailhaud *et al.*, 2006), however, the effect of perinatal exposure to an increased omega-6:omega-3 PUFA ratio remains unclear. Massiera *et al.* (2010) demonstrated that increasing omega-6 PUFA intake induced a gradual enhancement in fat mass over generations. However, the level of LA (~19% energy) and LA:ALA ratio (28:1) in their study were much higher than those found in typical Western diets, and the impact of different dietary fat levels was not evaluated. In addition, no studies to date have examined the effects of an increased maternal omega-6:omega-3 ratio on maternal, placental and fetal outcomes before birth.

The aim of the current study was to determine the effects of increasing the LA:ALA ratio (9:1 vs 1:1.5) in the diet of rats, on maternal weight gain, placental and fetal growth and the expression of lipogenic and adipokine genes in maternal and fetal liver and maternal adipose tissue. To determine any additive effects of altering the maternal dietary LA:ALA ratio, each diet was fed at either 18% fat w/w, to reflect dietary recommendations of fat intake (SACN, 2018), or at a higher fat content of 36% fat w/w.

3.3 Materials and Methods

3.3.1 Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Virgin female Wistar rats (n = 24; 75-100g; Charles River, UK) were housed on wood shavings in individually ventilated cages under a 12 hour light/12 hour dark cycle at a temperature of 20-22°C and had *ad libitum* access to food and water throughout the experiment. Animals were pair housed from the start of the experiment until mating; after confirmation of conception, animals were individually housed until completion of the experiment. Female rats were allowed to acclimatise to the unit for 1-2 weeks, during which time they were fed on standard laboratory chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK). After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status. The rats were then randomly assigned to one of 4 dietary groups, details of which are provided below. Animals were maintained on their allocated diet for a four week 'feed-in' period after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Average time in mating pairs was 2.5 days and all but one female conceived within their first oestrous cycle housed with the male. Female rats remained on their respective diets until day 20 of gestation (full term = 22 days) at which time rat dams were euthanised by CO_2 asphyxiation and cervical dislocation and fetuses by cervical dislocation and exsanguination for collection of maternal, fetal and placental tissues. All female rats were weighed and had feed intake measured daily throughout the experiment and protein intake was estimated based on the protein content of the experimental diets.

3.3.2 Diets

Diets were designed to provide either a high (9:1, high LA) or low (1:1.5 low LA) ratio of LA to ALA, achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were comparable in all diets, achieved by adjusting the amounts of coconut (SFA source) and macadamia (MUFA source) oils

in the diets. For each level of LA, diets containing either 18% or 36% fat by weight were developed. This resulted in four experimental diets; high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat; n=5-7 per dietary group). The lists of ingredients and final fatty acid composition of the four experimental diets are presented in Table 3.1 and Table 3.2 respectively. Protein content of experimental feeds was analysed using a Thermo Scientific nitrogen (N)/protein analyser (Flash EA1112). %N was directly measured and protein (%) was estimated under the assumption of a 16% N content of proteins.

3.3.3 Blood sample and tissue collection

Blood samples were collected from dams prior to the start of the experiment and after the 4 week 'feed-in' period (tail vein) and at day 20 of gestation (cardiac puncture following CO_2 asphyxiation and cervical dislocation). Truncal blood samples were also collected from two randomly selected male and 2 randomly selected female fetuses from each litter at this time. The whole blood samples (~30µl) were spotted onto PUFAcoat™ dried blood spot (DBS) collection paper (Liu *et al.*, 2014), allowed to dry at room temperature and were then stored at -20°C for subsequent fatty acid analysis. Maternal tissues were weighed and samples of liver and retroperitoneal adipose tissue were collected. All fetuses were weighed and sexed via measurement of anogenital distance. Fetal liver and placentas were weighed and samples of selected male placental and liver samples were collected. All tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until determination of gene expression by reverse transcriptase quantitative PCR (RT-qPCR). Male fetuses selected for collection of tissue samples also had tail samples collected for sex-genotyping by PCR for the SRY gene (McClive and Sinclair, 2001). Any samples found to be female or inconclusive (n=5) were not included in hepatic gene expression analyses.

3.3.4 Lipid extraction

Total lipids were extracted from male placental tissue with chloroform/isopropanol (2:1 v/v), an adapted method from Folch (Folch *et al.*, 1957). Briefly, ~300mg of crushed frozen placenta was weighed out and 2ml of 0.9% saline was added. The solution was homogenised on ice; 3ml of isopropanol was added and vortexed and the solution was left to stand for 5 minutes. After this, 6ml of chloroform was added and

the solution was shaken, centrifuged and the chloroform (bottom phase) containing the lipid was transferred into a fresh tube. This was dried under nitrogen and resuspended in chloroform/methanol (9:1 v/v) and 20µl of the sample was then spotted onto PUFAcoat™ DBS collection paper (Liu *et al.*, 2014), allowed to dry at room temperature and stored at -20°C for subsequent fatty acid analysis.

3.3.5 Fatty acid methylation and analysis

Fatty acid composition in maternal and fetal DBS were determined as previously described (Liu *et al.*, 2014). Briefly, whole DBS samples were directly transesterified with 2ml of 1%H_2SO_4 in methanol at 70°C for 3 hours. Samples were allowed to cool and the fatty acid methyl esters (FAME) were then extracted with heptane and transferred into a glass vial containing anhydrous sodium sulphate (Na_2SO_4) as a desiccant. Samples were separated and analysed by a Hewlett-Packard 6890 gas chromatograph (GC) equipped with a capillary column (30m x 0.25mm) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70; 0.25µm film thickness) which was fitted with a flame ionization detector (FID). Helium was utilised as the carrier gas and the split ratio was set at 20:1. Injector temperature was set at 250°C and FID temperature at 300°C. FAMEs were identified in unknown samples based on the comparison of retention times with an external lipid standard (Standard 463, Nu-check prep Inc., MN, USA) using Agilent Chemstation software. Individual fatty acid content was calculated based on peak area and response factors, normalised to total fatty acid content and expressed as a percentage of total fatty acids across lipid classes.

3.3.6 Isolation of RNA, cDNA synthesis and reverse transcription quantitative real-time PCR (RT-qPCR)

RNA was isolated from crushed snap-frozen samples of ~25mg of liver using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK). Adipose RNA was extracted using MagNA Lyser green beads and instrument (Roche Diagnostics Ltd.) in combination with the RNeasy Mini Kit (QIAGEN Ltd., UK) with the following modifications. Between 100-150mg of snap frozen tissue was homogenised using green beads in 600µl Buffer RTL (including β-mercaptoethanol); the sample was centrifuged for 3 minutes at 8000 rpm and the resulting infranatant was transferred into a fresh tube. The remaining tissue sample was homogenised in a further 350µl of

lysis buffer, centrifuged as before and this infranatant was combined with that from the initial extraction. This extract was centrifuged for 5 minutes at 13000 rpm, cleared lysate removed and mixed with an equal volume of 70% ethanol and transferred to an RNeasy column and from this point the standard manufacturer's protocol was applied. RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Lipogenic pathway and adipokine target genes included: peroxisome proliferator-activated receptor gamma (*Pparg*), sterol regulatory element-binding protein (variant 1c; *Srebf1*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*) and leptin (*Lep)*, with β-actin (*Actb*) as the housekeeper (Table 3). Adipocyte and hepatic gene expression was quantified using SYBR Green (Roche Diagnostics) in a Light-Cycler 480 (Roche Diagnostics). Samples were analysed against a standard curve of a serially diluted cDNA pool to produce quantitative data and expression was normalised to the housekeeping gene using LightCycler® 480 software (version 1.5.1) as previously described (Rhinn *et al.*, 2008). The expression of the housekeeper gene was not different between treatment groups.

3.3.7 Statistical analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). The effect of maternal dietary fatty acid ratio and maternal dietary fat content on maternal dependent variables was assessed using a two-way ANOVA, with dietary fat level and LA:ALA ratio as factors. Where longitudinal data were analysed, as with maternal feed, protein and energy intake as well as maternal bodyweight, the impact of maternal dietary fat level and LA:ALA ratio was analysed using a two-way repeated-measures ANOVA. Fetal data were analysed using a two-way ANOVA, separating males and females, with maternal dietary LA:ALA ratio and fat content as factors. The experiment was designed to detect a 0.5g difference in fetal weight with a power of 0.8; a value of $P<0.05$ was considered to be statistically significant and the dam was used as the unit of analysis.

3.4 Results

3.4.1 Maternal food intake

Dams receiving the 36% fat diets had a significantly lower food intake in the 4 weeks prior to mating independent of the LA:ALA ratio ($P<0.01$). During pregnancy, there was no effect of dietary fat content or fatty acid ratio on maternal food intake although, as expected, food intake in all groups increased as pregnancy progressed ($P<0.05$; Figure 3.1.A). Energy intake was similar between groups prior to pregnancy. During gestation, energy intake was higher in dams consuming the 36% fat diets, irrespective of dietary LA:ALA ratio ($P<0.01$). Similar to food intake, energy intake also increased with increasing gestational age in all dietary groups ($P<0.05$; Figure 3.1.B). Protein intake was lower in the 36% fat groups when compared to the 18% fat groups prior to mating ($P<0.001$) and throughout gestation ($P<0.01$). Protein intake was relatively consistent for the 4 weeks prior to mating but increased with increasing gestational age during pregnancy ($P<0.05$; Figure 3.1). There was no significant effect of dietary LA:ALA ratio on food, energy or protein intake at any time during the study.

3.4.2 Maternal fatty acid profile

There were no differences in the blood fatty acid profiles of the rats assigned to the different experimental diets before commencement of the dietary intervention, and the proportions of SFA, MUFA, omega-6 (Figure 3.2.A) and omega-3 PUFA (Figure 3.2.B) were all similar between groups. After 4 weeks on their respective diets, however, maternal fatty acid profiles were significantly different between treatments and largely reflected the composition of the experimental diets. Whole blood proportions of LA and AA were lower in the low LA compared to the high LA groups ($P<0.001$; Figure 3.2.C). Dams fed the low LA diets had four-fold higher ALA levels compared to the high LA groups, independent of dietary fat content ($P<0.001$). Whole blood proportions of DHA were also significantly higher ($P<0.01$) in dams fed the low LA diet, but the magnitude of this effect was relatively small (1.3-fold; Figure 3.2.D). The proportion of EPA was ten-fold higher and DPA two-fold higher in dams consuming the low LA diets. However, relative EPA and DPA levels were both lower in the low LA, 36% fat diet group compared to the low LA, 18% fat group, but were not different between the 36% and 18% fat diet levels in rats fed the high LA diets

(EPA: fat content, P<0.05; fatty acid ratio, P<0.001; interaction, P<0.05. DPA: fat content, P<0.01; fatty acid ratio, P<0.001; interaction, P<0.05; Figure 3.2.D). There was no effect of dietary fat content on the proportions of EPA and DPA at the end of the experiment. The remaining effects of both total fat level and dietary fat ratio on maternal fatty acid profile were, however, maintained until the end of the study (day 20 of gestation; Figure 3.2.E and F).

3.4.3 Maternal weight and body composition

Bodyweight immediately prior to commencement of feeding the experimental diets was not different between rats assigned to the four respective treatment groups (Figure 3.3). There was no effect of either LA:ALA ratio or dietary fat content on maternal weight prior to mating or throughout pregnancy (Figure 3.3) and maternal weight at day 20 of pregnancy was similar between groups (Figure 3.3; Table 3.4). Dams in the low LA groups had larger hearts relative to body weight at day 20 of gestation (P<0.05), but there were no differences in the relative weight of other major organs, including the liver, kidney, lung and brain, between groups (Table 3.4). There were no significant differences in the mass of the gonadal and retroperitoneal fat depots between experimental groups (Table 3.4). There were also no differences in the expression of any of the genes measured in the hepatic or adipose tissue between dietary groups (Table 3.4).

3.4.4 Placental fatty acid profile

The placental fatty acid profile at day 20 of gestation (Figure 3.4.A and B) was similar to dam whole blood fatty acid profile at this time point (Figure 3.2.E and F). Placentas of dams fed a high LA diet had 1.3 fold higher LA, AA and total omega-6 PUFA irrespective of dietary fat content (P<0.001; Figure 3.4.A). Conversely, total omega-3 PUFA (1.9 fold), ALA (5.5 fold) and EPA (6.5 fold) proportions were increased in placentas of dams consuming a low LA diet, regardless of dietary fat content (P<0.001). The relative levels of DPA in the placenta were higher in dams receiving a low LA diet but were also influenced by total dietary fat content, such that the proportion of DPA was lower in dams fed the 36% vs. 18% fat diets in the low LA ratio groups only (fat content, P<0.01; fatty acid ratio, P<0.001; interaction, P<0.01). DHA proportions were also influenced by both the fat content and LA:ALA ratio of

the diet, such that dams exposed to the low LA diets had higher placental DHA compared to those fed on high LA diets (P<0.001) and dams fed the 36% fat diet had higher relative levels of placental DHA compared to dams consuming either of the 18% fat diets (P<0.05; Figure 3.4.B). Placental total MUFA proportions were higher in the low LA compared to the high LA groups (P<0.001) but were not affected by dietary fat content. Placental SFA were lower in dams exposed to a low LA diet (P<0.05) as well as in dams exposed to a high (36%) fat diet (P<0.05), however the magnitude of this difference was very small (Figure 3.4.A).

3.4.5 Fetal whole blood fatty acid profile

There was no effect of sex on fetal fatty acid profile and so male and female samples were combined for subsequent analysis. Blood ALA (five-fold) and EPA (seven-fold) were higher in fetuses of dams exposed to the low LA diets. However, there was a significant interaction between maternal dietary LA:ALA ratio and maternal dietary fat content for these fatty acids, such that increasing dietary fat content from 18% to 36% fat was associated with relatively higher ALA (fat content, P<0.001; fatty acid ratio, P<0.001; interaction, P<0.001) and lower EPA levels (fat content, P=0.721; fatty acid ratio, P<0.001; interaction, P<0.05) in the low LA groups only. DPA (three-fold) and DHA (1.3-fold) blood proportions were also increased in fetuses of dams exposed to the low LA diets, independent of dietary fat content (P<0.001; Figure 3.4.D). Conversely, fetuses of dams consuming the low LA diets had relatively lower circulating LA (1.2-fold) and AA (1.4-fold) compared to fetuses of dams fed high LA diets (P<0.001; Figure 3.4.C). Interestingly, fetal LA proportions were also influenced by dietary fat content, such that fetuses of dams consuming the 36% fat diets had higher relative circulating LA (P<0.001) levels compared to fetuses of dams fed the 18% fat diet. Fetal blood MUFA were influenced by both maternal dietary LA:ALA ratio and dietary fat content. Thus, fetuses of dams exposed to a low LA diet exhibited elevated MUFA proportions compared to high LA groups (P<0.001) and fetuses of dams exposed to a 36% fat diet had proportionately lower circulating MUFA levels compared to fetuses of dams fed the 18% fat diets (P<0.001; Figure 3.4.C).

3.4.6 Fetal and placental weight and hepatic gene expression

The impact of maternal dietary fat content and LA:ALA ratio on placental and fetal weights was different for males and females. Thus, placental weight was higher in male, but not female, fetuses of dams consuming low LA diets ($P<0.05$). In females, however, fetuses of dams consuming the low LA diet were heavier ($P<0.01$) than those of dams fed the high LA diets, irrespective of dietary fat content; this effect was not seen in males. Despite these differences in fetal and placental weights, however, the fetal-placental weight ratio was not different between treatment groups in either males or females. Female, but not male, fetuses of dams consuming 36% fat diets also had smaller livers relative to bodyweight, independent of the dietary LA:ALA ratio ($P<0.05$; Table 3.5). Hepatic gene expression was not assessed in female fetuses, but hepatic mRNA expression of *Fasn, Lpl, Pparg* and *Srebf1* in male fetuses was not different between groups (Table 3.6).

3.5 Discussion

The results of the current study demonstrate that varying the fat content and LA:ALA ratio in the diet during pregnancy results in significant shifts in the fatty acid profile of the dam, placenta and fetus in late pregnancy with dietary LA:ALA ratio eliciting potentially sex-specific effects on placental and fetal weights. Importantly, these effects occurred in the absence of any impact of dietary LA:ALA ratio on maternal energy intake, bodyweight or fat deposition. Doubling the fat content of these experimental diets had the expected impact on maternal food and energy intake but was not associated with any significant alterations in maternal, placental or fetal weights or maternal fat deposition.

Maternal fatty acid profiles after 4 weeks on the experimental diets, and at the end of pregnancy, confirmed that the experimental diets had the desired effect on maternal fatty acid composition. Consistent with previous studies, increasing dietary ALA content had the greatest effect on maternal ALA and EPA proportions whereas relative DHA levels were only slightly higher than controls (Gibson *et al.*, 2013, Mantzioris *et al.*, 1994, Finnegan *et al.*, 2003, Wood *et al.*, 2015). However, despite the significant shifts in maternal fatty acid profiles, and differences in energy intake between dams in the 36% and 18% fat groups during gestation, we found no difference in maternal body

weight or fat deposition between these groups. This was unexpected as previous studies with comparable fat contents have reported significant increases in maternal bodyweight and fat mass in dams fed on diets containing higher amounts of total fat (Chalkley *et al.*, 2002, Buettner *et al.*, 2007). Consistent with previous studies (Keesey and Hirvonen, 1997), dams fed on the 36% fat diets in this study reduced their feed intake to compensate for the higher energy density of the feed and it may be that this adaptive response was sufficient to prevent excessive weight gain and fat deposition in dams fed the higher fat diet.

Our finding that placental and fetal fatty acid profiles were similar to those in the dams was consistent with our hypothesis that variations in maternal fatty acid profile would be reflected in the fetal compartment. Thus, our results confirm that shifts in maternal dietary fatty acid intake are associated with corresponding shifts in fetal fatty acid supply. It is, however, important to note that the relative proportions of some fatty acids, most notably LA and MUFA, in the fetal compartment also appeared to be influenced by total dietary fat content, independent of the dietary fat ratio. This effect was not observed to the same extent for maternal and placental fatty acid profiles and implies that not only the ratio, but also absolute amounts of fatty acids is a key determinant of placental fatty acid transfer. Our finding that fetal, but not maternal or placental, LA proportions were higher in the 36% fat vs 18% fat groups implies that placental LA transfer is favoured as dietary fat content increases. Placental capacity to store fatty acids as triglycerides has been well documented (Lewis and Desoye, 2017), and placental triglyceride levels are increased in conditions where maternal circulating triglyceride and fatty acid concentrations are high (Diamant *et al.*, 1982). However, this storage capacity is limited and, in cases of excess maternal dietary fat, has the potential to result in a greater proportion of fatty acids being transferred from maternal to fetal circulation. One possibility, therefore, is that the capacity of the placenta for LA storage is exceeded in the 36% fat groups in our current study and that this resulted in a greater amount of this fatty acid being transferred to the fetal compartment. In contrast to LA, however, fetal MUFA proportions in the current study were lower in the 36% fat compared to the 18% fat groups. Thus, the results of our study indicate that the effect of increased dietary fat content on placental fatty acid transfer varies substantially between individual fatty acids.

That the human placenta preferentially transfer specific fatty acids over others, following the hierarchy of AA>DHA>ALA>LA>oleic acid, has been previously demonstrated (Haggarty *et al.*, 1997). The findings from the present study suggest that this selective transfer effect may be exacerbated in the presence of an increased dietary fat load, such that the higher total amounts of LA and ALA in the 36% fat diets lead to greater transfer of LA and ALA to the fetal compartment, at the expense of MUFA, particularly oleic acid. If this is the case, it would imply that the quality of dietary fat intake during pregnancy may be more important, and thus have a greater potential to influence fetal growth and development, at higher levels of total dietary fat intakes. The fatty acid data in our study are expressed as a proportion of total lipids rather than absolute concentrations, and the relatively small proportionate change in fetal LA could translate into a much greater, and physiologically relevant, change in absolute concentrations.

In contrast to previous studies, we found no effect of maternal total fat intake on placental or fetal bodyweight, and our results in fact suggest that maternal LA:ALA ratio had a greater effect on these measures. The increased placental weight of male fetuses in the low LA groups suggests that an increase in maternal omega-3 status may potentially increase placental growth. One possibility for this increase in weight is that omega-3 fatty acids are preferentially deposited in the placenta. This, however, seems unlikely given that previous studies have suggested that while there is preferential placental uptake of DHA from the maternal circulation, the majority is transferred across the placenta to the fetal circulation (Ferchaud-Roucher *et al.*, 2017, Haggarty *et al.*, 1997). In addition, a considerable amount of omega-3 fatty acids (~75mg) would have to accumulate in the placenta to solely account for the difference in placental weight between high and low LA groups, making it unlikely that this mechanism could fully account for the observed increase in placental weight. An alternative possibility is that omega-3 PUFA, in particular DHA, acted to promote placental growth. Why the increased placental weight in our study was only seen in male placentas is not completely clear, however, female placental weight appeared to follow the same trajectory but did not reach statistical significance. Differences in gene expression between male and female placentas have been documented (Rosenfeld, 2015).

Given that the effects on placental weight only reached significance in males, it is interesting that maternal consumption of the low LA diet was associated with increased fetal weight only in females. Maternal omega-3 LCPUFA supplementation during pregnancy has been associated with increases in birth weight in human randomised controlled trials (Makrides *et al.*, 2006). However, this is generally considered to be secondary to an increase in the duration of gestation and therefore cannot explain the higher fetal weights observed in this study, since all fetuses were weighed at the same gestational age. There are, however, previous reports indicating that upregulation of omega-3 LCPUFA-responsive genes following maternal omega-3 LCPUFA supplementation is more pronounced in female compared to male placentas in humans, and is associated with increases in infant birth weight, but not placental weight (Sedlmeier *et al.*, 2014). Our finding of lower female fetal liver weight in the 36% fat groups is consistent with some (Krasnow *et al.*, 2011), but not all (Guo and Jen, 1995, McCurdy *et al.*, 2009), studies that have previously reported the effects of maternal high fat diets on neonatal liver weight. Whether the reduced liver weight in fetuses of dams consuming the high fat diet persists after birth, and whether it has functional consequences for hepatic metabolism, remain to be determined.

It was interesting that despite the significant changes in the fatty acid profiles of both dams and fetuses, no changes in maternal (hepatic and adipose tissue) or fetal (hepatic) expression of lipogenic genes were observed in this study. This was despite evidence from previous studies that omega-3 PUFA can suppress, while omega-6 PUFA can promote, expression of *Srebf1* and downstream lipogenic genes (Al-Hasani and Joost, 2005, Muhlhausler *et al.*, 2010a). It is possible, however, that any changes in maternal gene expression and corresponding increases in lipogenesis could be masked by the normal physiological adaptations that occur during pregnancy, particularly between day 19 and 20 of gestation when the pregnant rat experiences a marked increase in total body fat mass (Lopez-Luna *et al.*, 1991). Similarly, due to the immaturity of hepatic signalling pathways within the fetus, changes in gene expression occurring as a result of altered maternal dietary LA:ALA ratio may be too subtle to be detected.

In conclusion, we have demonstrated that both the total amount of fat and the ratio of LA and ALA in the maternal diet have significant effects on placental and fetal weight, supporting the hypothesis that the quality, as well as the quantity, of fat in the maternal

diet can impact on fetal growth and development. Importantly, the LA:ALA ratio in the current study was designed to reflect that of typical Western diets of many countries, and our results show that consuming this high LA diet was associated with alterations in placental and fetal weight in comparison to a LA:ALA ratio considered to be 'optimal' for human nutrition (Blasbalg *et al.*, 2011). The observed changes in placental and fetal weight may contribute to future metabolic and physiological function of the offspring, given the established association between reduced fetal and placental growth and increased future risk of obesity and poor metabolic health (Parsons *et al.*, 2001, Murtaugh *et al.*, 2003, Tian *et al.*, 2006). However, further studies are required in order to determine the longer-term impacts of maternal dietary LA:ALA ratio and total fat load on the growth, body composition and cardiometabolic health of the offspring. Human studies that have assessed the potential implication of a higher omega-6:omega-3 ratio and/or elevated maternal omega-6 intakes on growth, body composition or metabolic health of the children are also limited (Much *et al.*, 2013) and further studies (or re-evaluation of existing studies) are needed. The results of the current study have also demonstrated that at least some of the effects of maternal dietary fat load/LA:ALA ratio elicited sex-specific responses, which may in part be due to differences in the placental response to omega-3 LCPUFA between male and female placentas. Further research is required to assess the placental responses to shifts in maternal dietary fat content and composition in both males and females and the "sex of the placenta" should be considered in future studies.

Tables and Figures

Table 3.1 Ingredients in the experimental diets

Component	Amount (g/100g diet)			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Casein	16.0	16.0	16.0	16.0
Cornflour	31.8	20.0	31.8	20.0
Sucrose	16.0	10.0	16.0	10.0
Cellulose	15.6	15.6	15.6	15.6
Mineral Mix*	1.8	1.8	1.8	1.8
Vitamin Mix*	0.4	0.4	0.4	0.4
Choline Chloride	0.2	0.2	0.2	0.2
Methionine	0.4	0.4	0.4	0.4
Flaxseed Oil	0.9	1.7	4.2	8.5
Sunflower Oil	6.6	13.1	0.8	1.7
Macadamia Oil	6.9	13.8	9.5	19.0
Coconut Oil	3.4	6.9	3.2	6.3
Total Fat	17.8	35.6	17.8	35.5

LA, linoleic acid. *Composition of vitamin and mineral mixes based on AIN-76 formulation (American Institute of Nutrition, 1977).

Table 3.2 Fatty acid composition of the experimental diets

Fatty Acid	Amount (% of total lipids)			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Total SFA	20.7	20.3	21.1	20.0
Total TFA	0.1	0.1	0.1	0.1
Total MUFA	48.8	49.0	53.3	54.9
Total Omega-6 (LA only)	27.3	27.4	11.1	10.5
Total Omega-3 (ALA only)	3.1	3.2	14.4	14.4
Total PUFA	30.4	30.6	25.5	25.0
LA:ALA Ratio	9.0	9.0	0.75	0.75
ALA %Energy	1.2	2.0	5.6	9.1
LA %Energy	10.5	17.3	4.3	6.7

LA, linoleic acid; SFA, saturated fatty acids; TFA, trans fatty acids; MUFA, monounsaturated fatty acids; ALA, alpha-linolenic acid; PUFA, polyunsaturated fatty acids.

Table 3.3 Primer sequences used for the determination of gene expression by RT-qPCR

Target Genes	Sequence (5'-3')	Amplicon Size	Accession Number
Fasn	FWD: TGCTCCCAGCTGCAGGC REV: GCCCGGTAGCTCTGGGTGTA	107	NM_017332
Lpl	FWD: TTCCTGGATTAGCAGACTCTGTGT REV: TCCTGTCACCGTCCATCCAT	89	NM_012598
Pparg	FWD: CTCAGTGGAGACCGCCCA REV: CAGGGCCTGCAGCAGGT	75	NM_013124
Srebf1	FWD: GATTGCACATTTGAAGACATGCTT REV: CCTGTCTCACCCCCAGCATA	95	NM_001276708
Lep	FWD: AGACCATTGTCACCAGGATCAAT REV: CCCGGGAATGAAGTCCAAA	89	NM_013076
Actb	FWD: GACCCAGATCATGTTTGAGACCTT REV: AGAGGCATACAGGGACAACACA	79	NM_031144

Fasn, fatty acid synthase; *Lpl*, lipoprotein lipase; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf1*, sterol regulatory element-binding protein 1; *Lep*, leptin; *Actb*, beta-actin.

Table 3.4 Maternal organ weight and gene expression

	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Bodyweight (g)	376.4 ± 4.65	384.41 ± 11.00	384.34 ± 16.90	353.63 ± 9.55
Heart (%BW)	0.25 ± 0.01[a]	0.26 ± 0.01[a]	0.28 ± 0.02[b]	0.28 ± 0.01[b]
Lungs (%BW)	0.37 ± 0.05	0.36 ± 0.03	0.38 ± 0.05	0.36 ± 0.04
Kidney (%BW)	0.53 ± 0.02	0.50 ± 0.01	0.53 ± 0.02	0.54 ± 0.02
Liver (%BW)	4.01 ± 0.14	3.95 ± 0.07	4.10 ± 0.10	3.94 ± 0.10
Brain (%BW)	0.47 ± 0.02	0.46 ± 0.01	0.50 ± 0.03	0.53 ± 0.02
Gonadal Fat (%BW)	2.95 ± 0.03	2.38 ± 0.18	2.33 ± 0.18	3.04 ± 0.58
Retroperitoneal Fat (%BW)	1.18 ± 0.17	1.18 ± 0.17	1.18 ± 0.011	1.79 ± 0.24
Liver mRNA Expression				
Fasn	5.51 ± 1.33	2.83 ± 0.59	6.08 ± 2.31	4.24 ± 1.21
Lpl	3.13 ± 1.00	2.39 ± 0.75	2.52 ± 0.80	1.74 ± 0.71
Pparg	5.81 ± 1.85	2.30 ± 0.77	3.38 ±0.78	2.09 ± 0.73
Srebf1	1.33 ± 0.31	1.20 ± 0.22	1.23 ± 0.50	2.67 ± 1.14
Retroperitoneal Fat mRNA Expression				
Fasn	0.12 ± 0.05	0.07 ± 0.04	0.15 ± 0.05	0.13 ± 0.07
Lpl	2.18 ± 0.66	1.82 ± 0.81	2.18 ± 0.72	1.48 ± 0.16
Pparg	1.15 ± 0.47	1.40 ± 0.59	1.76 ± 0.48	1.03 ± 0.17
Srebf1	1.17 ± 0.40	1.16 ± 0.41	1.38 ± 0.42	1.11 ± 0.13
Lep	2.59 ± 0.67	2.73 ± 0.99	2.31 ± 0.69	1.83 ± 0.34

Data are presented as mean values ± SEM (n=4-6 per group). LA, linoleic acid; BW, bodyweight; mRNA, messenger ribonucleic acid; *Fasn*, fatty acid synthase; *Lpl*, lipoprotein lipase; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf1*, sterol regulatory element-binding protein 1; *Lep*, leptin. Mean values with unlike superscript letters were significantly different ($P<0.05$).

Table 3.5 Fetal and placental weights and fetal organs

	Sex	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
Fetal bodyweight (g)	Male	3.33 ± 0.04	3.25 ± 0.08	3.55 ± 0.09	3.32 ± 0.09
	Female	3.01 ± 0.06[a]	2.95 ± 0.09[a]	3.36 ± 0.12[b]	3.21 ± 0.08[b]
Placenta weight (g)	Male	0.50 ± 0.02[a]	0.52 ± 0.03[a]	0.56 ± 0.03[b]	0.61 ± 0.02[b]
	Female	0.48 ± 0.02	0.48 ± 0.04	0.52 ± 0.02	0.51 ± 0.03
Fetal liver weight (% BW)	Male	7.43 ± 0.42	6.52 ± 0.11	6.81 ± 0.58	6.97 ± 0.25
	Female	7.51 ± 0.38[a]	6.99 ± 0.29[b]	7.73 ± 0.22[a]	6.86 ± 0.28[b]
Fetal-placental ratio	Male	6.79 ± 0.24	6.37 ± 0.33	6.51 ± 0.41	5.99 ± 0.41
	Female	6.38 ± 0.32	6.35 ± 0.48	7.72 ± 0.96	6.13 ± 0.36

Data are presented as mean values ± SEM (n=4-6 per group). LA, linoleic acid; BW, bodyweight. Mean values with unlike superscript letters were significantly different ($P<0.05$).

Table 3.6 Fetal liver gene expression

	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Fasn	0.59 ± 0.19	0.30 ± 0.03	0.37 ± 0.06	0.49 ± 0.13
Lpl	1.79 ± 0.55	1.41 ± 0.27	1.11 ± 0.33	1.17 ± 0.46
Pparg	0.36 ± 0.12	0.42 ± 0.05	0.34 ± 0.07	0.48 ± 0.16
Srebf1	1.21 ± 0.61	0.65 ± 0.30	0.27 ± 0.04	0.82 ± 0.30

Data are presented as mean values ± SEM (n=4-6 per group). LA, linoleic acid; *Fasn,* fatty acid synthase; *Lpl,* lipoprotein lipase; *Pparg,* peroxisome proliferator-activated receptor gamma; *Srebf1,* sterol regulatory element-binding protein 1.

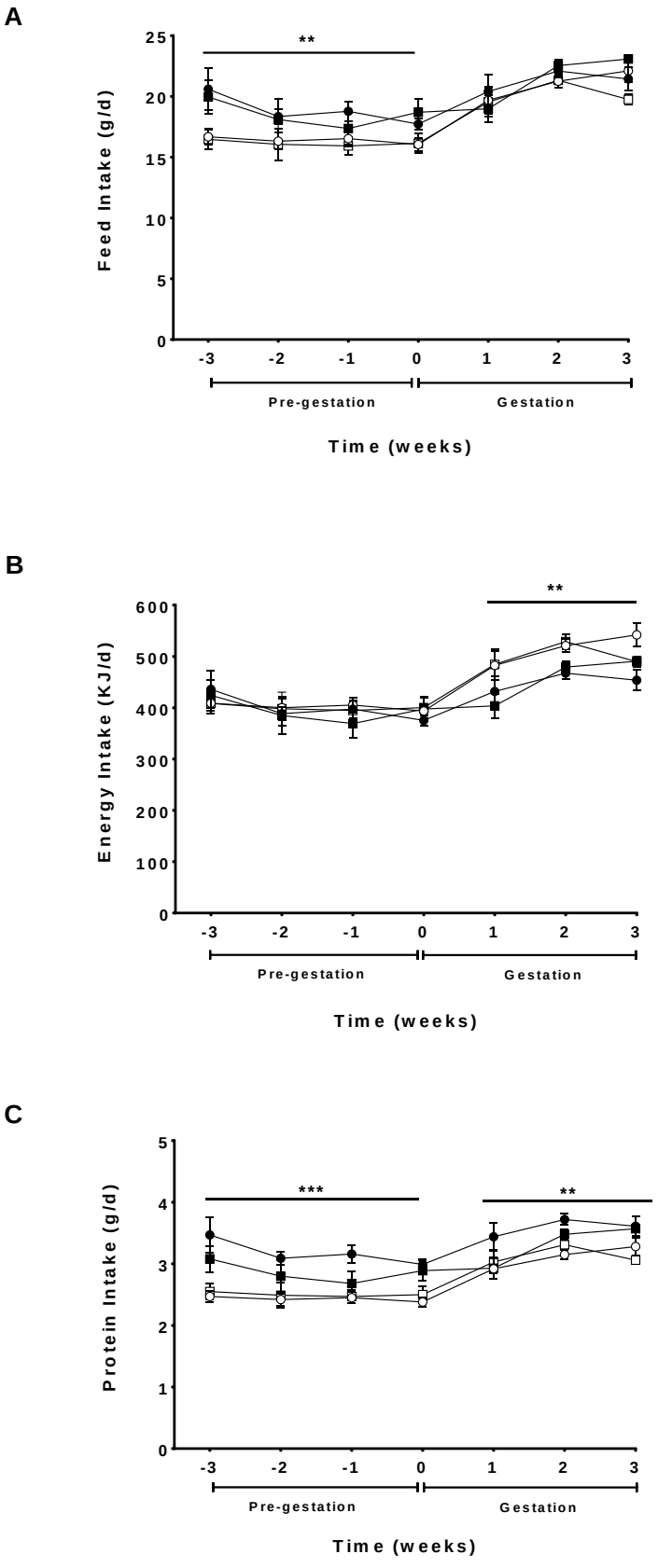
Figure 3.1 Maternal average daily (A) food intake, (B) energy intake and (C) protein intake before mating and up to day 20 of gestation fed on either a high LA (18% fat) diet (closed circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) and a low LA (36% fat) diet (open squares). Values are means ± SEM and n=5-7 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way repeated measures ANOVA. * indicates a significant effect of dietary fat content (** $P<0.01$, *** $P<0.001$).

Figure 3.2 Maternal whole blood fatty acids profile at (A/B) baseline (C/D) after 4 weeks on experimental diet and (E/F) at day 20 of gestation. Values are means ± SEM and n=5-7 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$). † indicates a significant interaction between dietary fat content and LA:ALA ratio ($P<0.05$).

Figure 3.3 Maternal weight gain of rats during pre-feeding and up to day 20 of gestation fed on either a high LA (18% fat) diet (closed circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) and a low LA (36% fat) diet (open squares). Values are means ± SEM and n=5-7 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way repeated measures ANOVA and no significant differences were found between groups.

Figure 3.4 Placental (A/B) and fetal whole blood (C/D) fatty acid profile at gestational day 20. Values are means ± SEM and n=10-14 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$). † indicates a significant interaction between dietary fat content and LA:ALA ratio ($P<0.05$).

Figure 3.1


A
**
Feed Intake (g/d)
25
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10
5
0
-3
-2
-1
0
1
2
3
Pre-gestation
Gestation
Time (weeks)
B
**
Energy Intake (KJ/d)
600
500
400
300
200
100
0
-3
-2
-1
0
1
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Pre-gestation
Gestation
Time (weeks)
C
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Protein Intake (g/d)
5
4
3
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-3
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-1
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1
2
3
Pre-gestation
Gestation
Time (weeks)

Figure 3.2

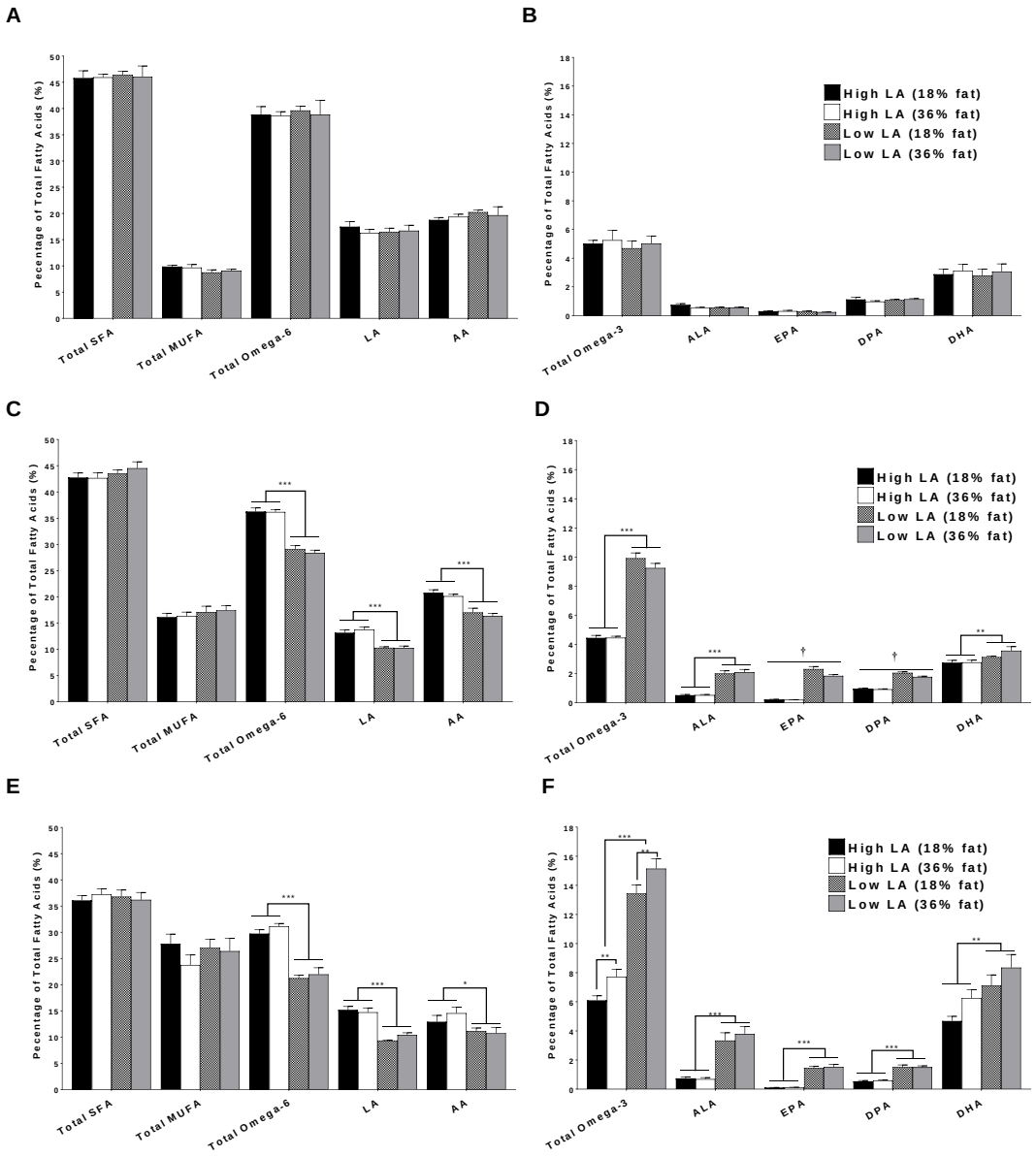

A
B
C
D
E
F
Pecentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)

Figure 3.3

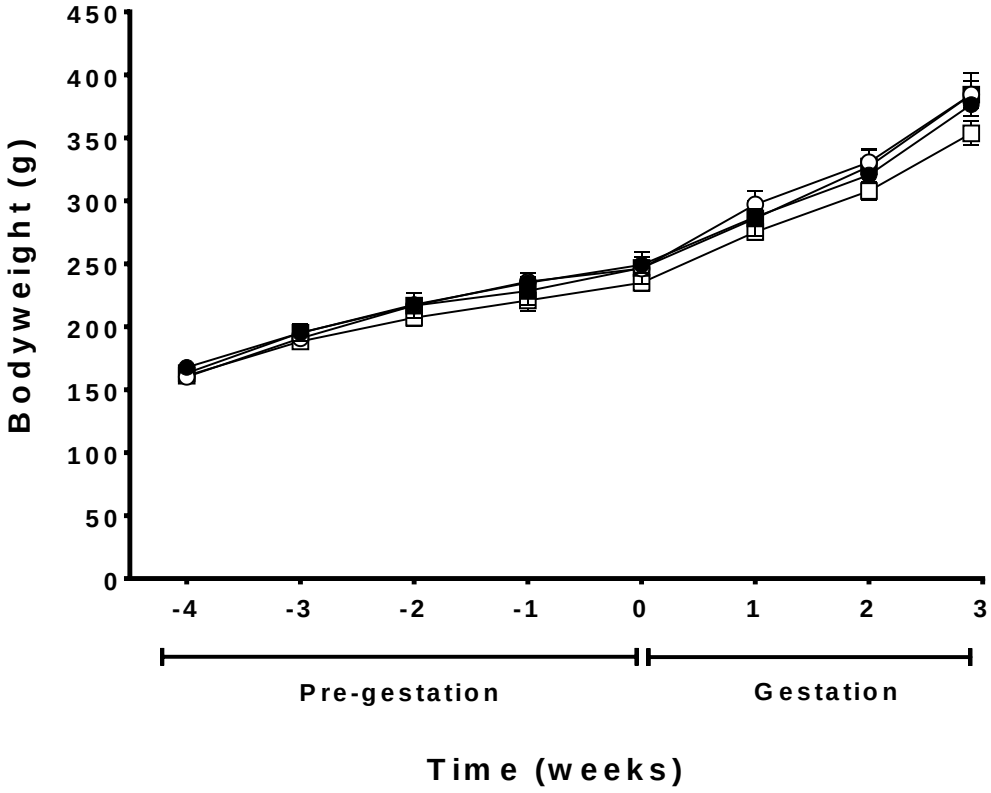
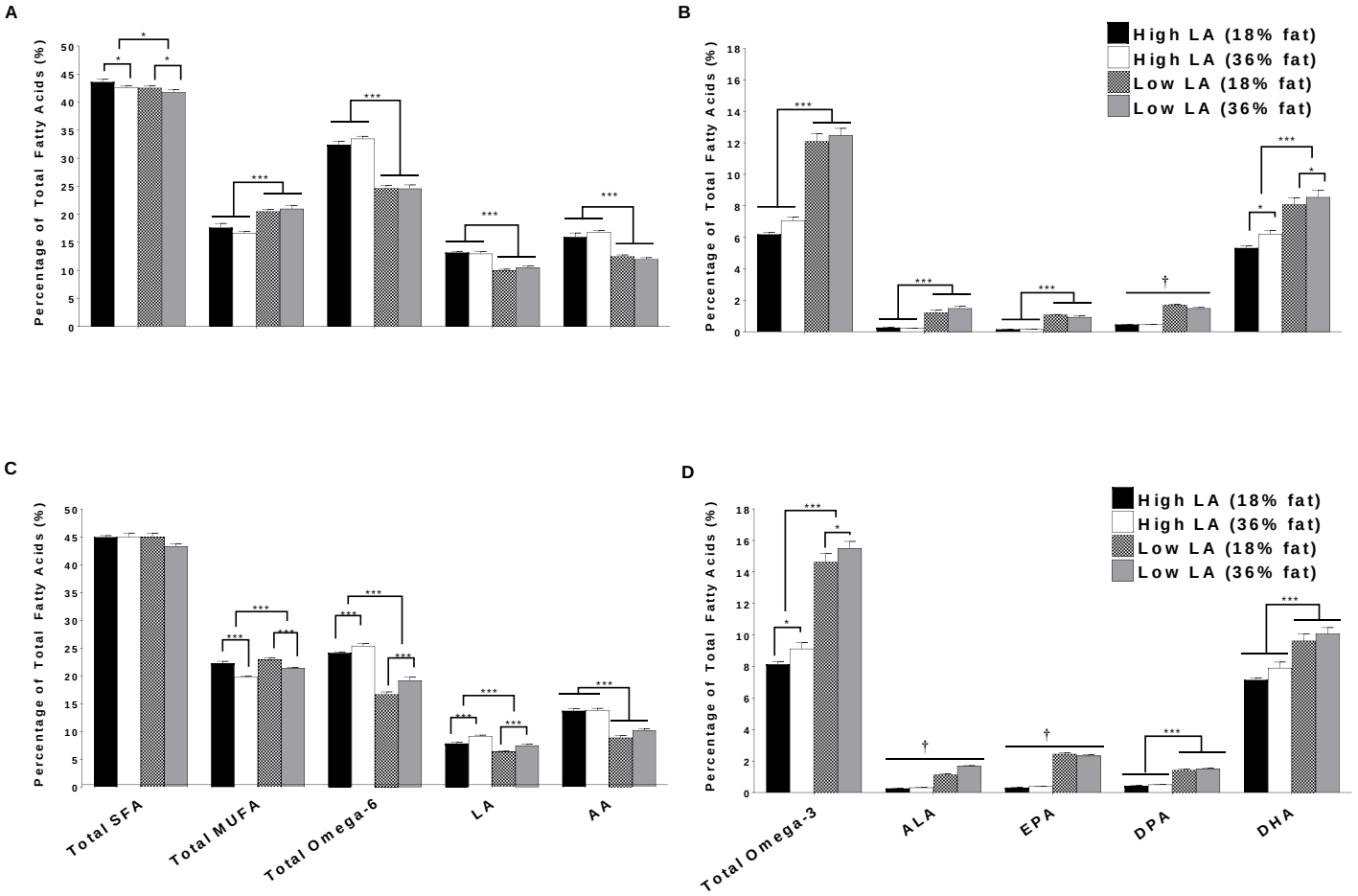

Bodyweight (g)
450
400
350
300
250
200
150
100
50
0
-4
-3
-2
-1
0
1
2
3
Pre-gestation
Gestation
Time (weeks)

Figure 3.4


A
B
C
D
Percentage of Total Fatty Acids (%)
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA

4 MANUSCRIPT 2: THE EFFECT OF MATERNAL DIETARY FAT CONTENT AND OMEGA-6 TO OMEGA-3 RATIO ON OFFSPRING GROWTH AND HEPATIC GENE EXPRESSION IN THE RAT

Draycott, SAV, George, G, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). The effect of maternal dietary fat content and omega-6 to omega-3 ratio on offspring growth and hepatic gene expression in the rat. *British Journal of Nutrition* (accepted manuscript).

Author contributions can be found in Appendix C and the submitted article PDF can be found in Appendix E.

4.1 Abstract

Abstract

Omega-6 fatty acids have been shown to exert pro-adipogenic effects whereas omega-3 fatty acids appear to work in opposition. Increasing intakes of LA (linoleic acid; omega-6) vs ALA (alpha-linolenic acid; omega-3) in Western diets has led to the hypothesis that consumption of this diet during pregnancy may be contributing to adverse offspring health. This study investigated the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) compared to a proposed 'ideal' ratio (~1:1.5), at two total fat levels (18% vs 36% fat w/w), on growth and lipogenic gene expression in the offspring. Female Wistar rats were assigned to one of the four experimental groups throughout gestation and lactation. Offspring were culled at 1 and 2 weeks of age for blood and tissue sample collection. Offspring of dams consuming a 36% fat diet were ~20% lighter than those exposed to an 18% fat diet ($P<0.001$). Male, but not female, liver weight at 1 week was ~13% heavier, and had increased glycogen ($P<0.05$), in offspring exposed to high LA ($P<0.01$). Hepatic expression of lipogenic genes suggested an increase in lipogenesis in male offspring exposed to a 36% fat maternal diet and in female offspring exposed to a low LA diet, via increases in the expression of *Fasn* and *Srebf1*. Sexually dimorphic responses to altered maternal diet appeared to persist until two weeks-of-age. In conclusion, whilst maternal total fat content predominantly affected offspring growth, fatty acid ratio and total fat content had sexually dimorphic effects on offspring liver weight and composition.

4.2 Introduction

Accumulating evidence suggests that the nutritional environment experienced by an individual during fetal and early infant development has long-lasting impacts on their metabolic health (Langley-Evans, 2015). In the context of the global epidemic of obesity and nutritional excess, there has been considerable interest in determining the effects of maternal overnutrition on the metabolic health of the offspring. The majority of these studies have utilised animal models and have consistently reported that maternal high-fat feeding during pregnancy has detrimental effects on the metabolic health of both the mother and her offspring (Ribaroff *et al.*, 2017, Ainge *et al.*, 2011). As a result, excess maternal fat consumption has been implicated as a key contributor to metabolic programming of long-term health and disease risk.

There is increasing evidence, however, that the impact of a high-fat diet on the metabolic health of the offspring depends not only on the amount of fat in the diet, but also on the fatty acid composition (Muhlhausler and Ailhaud, 2013, Draycott *et al.*, 2019). There has been particular interest in the role of two classes of polyunsaturated fatty acids (PUFA), due to the substantive increase in the amounts of omega-6 PUFA, predominately linoleic acid (LA), being consumed in the diets of many Western countries over the past few decades (Blasbalg *et al.*, 2011, Sioen *et al.*, 2017). This increase in the intake of LA has not been accompanied by substantial changes in the consumption of omega-3 PUFA such as alpha-linolenic acid (ALA) and has therefore resulted in increases in the ratio of omega-6:omega-3 PUFA consumed in the diets of many Western countries (Ailhaud *et al.*, 2006, Blasbalg *et al.*, 2011).

The increasing dominance of omega-6 over omega-3 PUFA in modern Western diets has considerable biological significance, since the omega-6 and omega-3 fatty acid families utilise the same enzymes for production of longer chain bioactive derivatives such as arachidonic acid (AA; omega-6), eicosapentaenoic acid (EPA; omega-3), docosapentaenoic acid (DPA; omega-3) and docosahexaenoic acid (DHA; omega-3), and also compete for incorporation into cell membranes. As a result, excess consumption of LA leads to a decrease in the production and incorporation of omega-3 fatty acids through simple substrate competition, and this effect is exacerbated when total dietary PUFA is high (Tu *et al.*, 2010, Gibson *et al.*, 2013). The omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) derivatives also have

opposing physiological actions, with those from the omega-3 family predominately exhibiting anti-inflammatory properties (for example via the suppression of the pro-inflammatory transcription factor nuclear factor kappa B and activation of the anti-inflammatory transcription factor peroxisome proliferator activated receptor γ (Calder, 2015)) and those from the omega-6 family exhibiting more pro-inflammatory and pro-adipogenic properties (Massiera *et al.*, 2003). This has led to the hypothesis that the increasing ratio of omega-6 to omega-3 fatty acids in modern Western diets may have negative effects on conditions characterised by low-grade inflammation, including obesity and the metabolic syndrome, and may potentially be contributing to an intergenerational cycle of obesity (Ailhaud *et al.*, 2006).

Data from observational studies in humans and animal models provide supporting evidence that suggests high intakes of omega-6 PUFA during pregnancy could have negative effects on metabolic health of the progeny (Massiera *et al.*, 2010, Korotkova *et al.*, 2002, Much *et al.*, 2013). However, the results of these studies have not been consistent. The results of pre-clinical studies are also limited by the use of diets with much higher omega-6:omega-3 PUFA ratios and/or absolute PUFA contents than those encountered in typical human diets. Furthermore, offspring often continue to have access to the same diet as their mother so that any effects observed cannot be clearly attributed to dietary fatty acid exposure during the gestation and lactation periods (Muhlhausler *et al.*, 2011b, Ibrahim *et al.*, 2009, Muhlhausler *et al.*, 2011a). The aim of this study, therefore, was to investigate the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) (Blasbalg *et al.*, 2011), compared to a proposed 'ideal' ratio of ~1:1.5 (Gibson *et al.*, 2011, Lands, 2000) on growth and lipogenic gene expression in the offspring. Since total dietary PUFA intake also influences PUFA metabolism (Tu *et al.*, 2010, Gibson *et al.*, 2013), we also investigated the effect of feeding each dietary fat ratio at either 18% fat w/w (in line with dietary recommendations (SACN, 2018)) or at a higher fat level of 36% fat w/w. A rat model was utilised to achieve the study objectives by allowing for tight control of dietary manipulation as well as invasive end points.

4.3 Materials and Methods

4.3.1 Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Virgin female Wistar rats (n=30; 75-100g; Charles River, UK) were housed on wood shavings in individually ventilated cages under a 12 hour light/12 hour dark cycle at a temperature of 20-22°C and had *ad libitum* access to food and water throughout the experiment. Female rats were allowed to acclimatise to the unit for 1-2 weeks, during which time they were fed standard laboratory chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK). After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status. The rats were then randomly assigned to one of 4 dietary groups (details provided below). Animals were maintained on their allocated diet for a four week 'pre-feeding' period, after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Animals were housed in individual cages and remained on their respective diets throughout pregnancy and lactation.

Litters were standardised to 8 pups within 24 hours of birth (4 males and 4 females, where possible). At 1 and 2 weeks of age, one randomly selected male and one randomly selected female from each litter were culled via cervical dislocation and exsanguination for blood and tissue collection. At 3 weeks of age, the remaining offspring were weaned and dams were then euthanised by CO_2 asphyxiation and cervical dislocation for collection of maternal blood and tissues. All dams were weighed and had feed intake measured daily throughout the experiment and offspring bodyweight was measured weekly.

4.3.2 Diets

Diets were designed to provide either a high (9:1, high LA) or low (1:1.5, low LA) ratio of LA (cis/cis isomer) to ALA, achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The levels of saturated and monounsaturated fatty acids were comparable in all diets, achieved by adjusting the amounts of coconut (saturated fat source) and macadamia (monounsaturated fat

source) oils in the diets. For each level of LA, diets were developed to contain either 18% fat (w/w), in line with government recommendations (SACN, 2018), or 36% fat (w/w) to highlight any additive effects (38.6 vs 63.5% of dietary energy respectively). This resulted in four experimental diets; high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat; n=6-9 per dietary group). The list of ingredients and final fatty acid composition of the four experimental diets have been reported previously (Draycott *et al.*, 2019).

4.3.3 Blood sample and tissue collection

Blood samples were collected from dams prior to the start of the experiment and after the 4 week 'feed-in' period (tail vein sample) and at the end of lactation (via cardiac puncture following CO_2 asphyxiation and cervical dislocation). Truncal blood samples were also collected from one randomly selected male and one randomly selected female at 1 and 2 weeks of age. In all cases, samples of whole blood (~30µl) from non-fasted animals were spotted onto PUFAcoat™ dried blood spot (DBS) collection paper (Liu *et al.*, 2014), allowed to dry at room temperature and stored at -20°C for subsequent fatty acid analysis. Maternal tissues were weighed and samples of whole liver, retroperitoneal and gonadal adipose tissues collected. Offspring body and organ weights were measured and whole liver samples were collected from one randomly selected male and female pup at both time points. At 2 weeks of age, samples of gonadal and retroperitoneal fat were also collected from one male and one female pup per litter. All tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until determination of gene expression by quantitative reverse transcriptase PCR (qRT-PCR).

4.3.4 Fatty acid methylation and analysis

Fatty acid composition in maternal and fetal blood was determined as previously described (Liu *et al.*, 2014). Briefly, whole DBS samples were directly transesterified with 2ml of 1% H_2SO_4 in methanol and the fatty acid methyl esters (FAME) were extracted with heptane. Samples were separated and analysed by a Hewlett-Packard 6890 gas chromatograph (GC) equipped with a capillary column (30m x 0.25mm) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70; 0.25µm film thickness) which was fitted with a flame ionization detector (FID). FAMEs were

identified in unknown samples based on the comparison of retention times with an external lipid standard (Standard 463, Nu-check prep Inc., MN, USA) using Agilent Chemstation software (Agilent Technologies Australia Pty Ltd). Individual fatty acid content was calculated based on peak area and response factors normalised to total fatty acid content and expressed as a percentage of total fatty acids.

4.3.5 Isolation of RNA and cDNA synthesis and reverse transcription quantitative real-time PCR (qRT-PCR)

RNA was isolated from crushed snap-frozen samples of ~25mg of liver using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK). Adipose RNA was extracted, after homogenisation of ~100mg of tissue with MagNA Lyser green beads and instrument (Roche Diagnostics Ltd.), using the RNeasy Mini Kit (QIAGEN Ltd., UK). RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Lipogenic pathway and adipokine target genes included were chosen based on previous data from our laboratory that indicated that these genes were sensitive to changes in the maternal diet (Erhuma *et al.*, 2007b) and included; peroxisome proliferator-activated receptor gamma (*Pparg*), sterol regulatory element-binding protein (variant 1c; *Srebf1*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*) and leptin (*Lep*), with β-actin (*Actb*) as the housekeeper. Primer efficiency ranged from 85%-108% and sequences have previously been published elsewhere (Draycott *et al.*, 2019). Adipocyte and hepatic gene expression was quantified using SYBR Green (Roche Diagnostics) in a Light-Cycler 480 (Roche Diagnostics). Samples were analysed against a standard curve of a serially diluted cDNA pool to produce quantitative data and expression was normalised to the housekeeping gene using LightCycler® 480 software (version 1.5.1) as previously described (Rhinn *et al.*, 2008). The expression of the housekeeper gene was not different between treatment groups.

4.3.6 Determination of liver DNA, protein and glycogen content

For determination of DNA and protein content of liver samples, approximately 100mg of frozen crushed sample was added to 1ml of 0.05M trisodium citrate buffer. Samples were homogenised and centrifuged at 2500rpm for 10 minutes at 4°C. Supernatant was used for further analyses. DNA concentration (µg/ml) was measured using a Hoechst fluorometric method and protein concentration (mg/ml), modified for a 96 well plate format, was measured as described by Lowry *et al.* (1951). Measurements were normalised to the exact amount of tissue used for measurements. Liver glycogen was measured using the Colorimetric Glycogen Assay Kit II (Abcam Ltd.) according to manufacturer's instructions.

4.3.7 Statistical analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). The effect of maternal dietary fatty acid ratio and maternal dietary fat content on maternal dependent variables was assessed using a two-way ANOVA, with dietary LA:ALA ratio and dietary fat content as factors and dams were used as the unit of analysis. Where longitudinal data were analysed, as with maternal feed, protein and energy intakes, the impact of maternal dietary LA:ALA ratio and maternal dietary fat content was analysed using a two-way repeated-measures ANOVA. Offspring data were analysed using a two-way ANOVA, with maternal dietary LA:ALA ratio and fat content as factors; where there was no overall effect of sex, male and female offspring data were combined. Where data were not normally distributed, analyses were performed on log10 transformed data. A value of $P<0.05$ was considered to be statistically significant.

4.4 Results

4.4.1 Maternal dietary intakes

There were no differences in feed intake of dams between treatment groups before or during pregnancy. During lactation, dams receiving the 36% fat diets had a lower average daily feed intake than those receiving the 18% fat diets, irrespective of dietary LA:ALA ratio ($P<0.001$; Figure 4.1.A). Energy intake was similar between groups throughout the experiment (Figure 4.1.B). Protein intake prior to and during pregnancy

was affected by both dietary LA:ALA ratio and fat content (P<0.05; Figure 4.1.C), however, these effects were small and inconsistent. During lactation, protein intake was affected by dietary fat content only (P<0.001; Figure 4.1.C), such that mothers receiving 36% fat diets consumed 24% less protein on average compared to those consuming 18% fat diets, irrespective of dietary LA:ALA ratio. As expected, all dams consumed more food, energy and protein during lactation than before and during pregnancy regardless of dietary group (P<0.001).

4.4.2 Maternal fatty acid profile

There were no differences in the proportions of either saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), omega-6 (Figure 4.2.A) or omega-3 PUFA (Figure 4.2.B) in whole blood samples collected from the dams prior to the commencement of dietary intervention. After 4 weeks on their respective diets, the blood fatty acid profiles were significantly different between treatment groups and largely reflected the composition of the experimental diets. Thus, dams fed on high LA diets had higher proportions of LA (1.2 fold) and AA (1.4 fold) compared to those consuming a low LA diet (P<0.001; Figure 4.2.C). Conversely, dams fed the low LA diets had a 5.5 fold higher proportion of ALA and an 8.5 fold higher proportion of EPA compared to those consuming a high LA diet (P<0.001; Figure 4.2.D). These changes were independent of the total fat content of the diet. DPA and DHA levels after the 4 week pre-feeding period were influenced by both dietary LA:ALA ratio and total fat content. Thus, the relative proportions of DPA were higher in dams fed the low LA compared to high LA diets (P<0.001), and marginally higher in dams consuming the 18% vs 36% fat diets (P<0.05). DHA proportions were also higher in the low LA group (P<0.001) but, unlike DPA, were modestly but significantly higher in dams consuming the 36% fat vs 18% fat diets (P<0.05; Figure 4.2.D). Total blood MUFA proportions were higher (1.3-fold) in dams consuming the low LA diet, irrespective of dietary fat content (P<0.001; Figure 4.2.D).

The blood fatty acid profile of the dams at the end of lactation, after a further 6 weeks on their respective experimental diets, were similar to those observed after the first 4 weeks of dietary intervention. A notable difference, however, was that at this time point, relative proportions of DHA, as a percentage of total lipids, were not different between dietary groups (Figure 4.2.F). LA (1.5-fold), AA (1.8-fold) and total omega-

6 (1.5-fold) were all higher in dams consuming a high LA diet irrespective of dietary fat content ($P<0.001$; Figure 4.2.E). Conversely, total omega-3 levels were 3-fold higher in dams consuming a low LA diet, irrespective of dietary fat content ($P<0.001$). The proportions of ALA were also higher in the groups consuming the low LA diets and in rats consuming the 36% vs 18% fat diets in the low LA group only ($P<0.05$; Figure 4.2.F). DPA proportions were higher in the groups consuming the low LA diets, however, unlike ALA, DPA proportions were lower, rather than higher, in dams consuming the 36% fat diets in the low LA group only ($P<0.001$; Figure 4.2.F). EPA proportions were higher in groups consuming a low LA diet compared to those consuming a high LA diet ($P<0.001$; Figure 4.2.F). EPA proportions were also affected by total dietary fat content, and were lower in dams consuming a 36% fat diet compared to an 18% fat diet ($P<0.001$; Figure 4.2.F). Maternal blood total MUFA levels at the end of lactation were 1.4-fold higher in the dams consuming a low LA diet irrespective of dietary fat content ($P<0.001$; Figure 4.2.E).

4.4.3 Maternal weight, body composition and gene expression

There were no significant differences in dam bodyweight between dietary groups prior to the commencement of the dietary intervention or at any time during the experiment (data not shown). Dams consuming the 36% fat diets had heavier lungs relative to bodyweight at the end of lactation compared to those consuming the 18% fat diets, independent of the LA:ALA ratio ($P<0.05$). There were no differences in the relative weight of the heart, liver, brain, kidney, gonadal or retroperitoneal fat pads between experimental groups (Table 4.1).

Analysis of mRNA expression of lipogenic genes indicated that hepatic (3-fold) and gonadal fat (7-fold) expression of *Fasn* was higher in dams consuming an 18% fat diet, compared to those on a 36% fat diet, irrespective of dietary fatty acid ratio ($P<0.01$). The mRNA expression of *Lpl*, *Pparg* and *Srebf1* was not, however, affected by either dietary fat content or ratio in either hepatic or gonadal fat tissues (Table 4.1). Expression of leptin mRNA in gonadal adipose tissue was not significantly different between treatment groups.

4.4.4 Birth outcomes and offspring bodyweights

There were no differences between dietary groups in terms of litter size or sex ratio of pups (Table 4.2). Birth weight was lower in offspring of dams fed a 36% fat vs 18% fat diets, independent of the dietary LA:ALA ratio (Table 4.2). The lower body weight in offspring of dams fed the 36% fat diet persisted during the sucking period such that offspring of dams fed the 36% fat diets remained lighter than offspring of dams fed on 18% fat diets at both 1 and 2 weeks of age; again this was independent of dietary LA:ALA ratio ($P<0.001$; Table 4.3).

4.4.5 Offspring fatty acid profile

At 1 week of age, proportions of AA (2.1 fold) were lower in the offspring of the low LA compared to high LA dams ($P<0.001$), and in offspring of dams consuming the 36% fat vs 18% fat diets (1.4 fold; $P<0.001$; Figure 4.3.A). Blood ALA proportions were 5.9 fold higher in offspring of dams in the low LA groups compared to high LA groups ($P<0.001$; Figure 4.3.B). Offspring EPA and DPA proportions were also higher in the low LA group compared to the high LA group. Blood EPA was also influenced by total dietary fat content, but only in offspring of dams fed the low LA diet, in which EPA levels were lower in offspring of dams fed the 36% fat diets compared to the 18% fat diets (EPA, $P<0.001$; DPA, $P<0.01$; Figure 4.3.B). DHA proportions were not different between groups at 1 week of age (Figure 4.3.B). MUFA proportions were higher (1.2-fold) in offspring of dams in the low LA groups ($P<0.001$), consistent with the pattern in maternal blood. However, unlike maternal MUFA, offspring MUFA levels were also affected by maternal dietary fat content and were 1.2-fold higher in offspring of dams fed the 36% fat vs 18% fat diets ($P<0.001$; Figure 4.3.A). At 1 week of age offspring of dams in the 36% fat diet groups also had lower blood proportions of SFA, irrespective of LA:ALA ratio of the maternal diet ($P<0.01$; Figure 4.3.A).

The fatty acid profiles of the offspring at 2 weeks of age were similar to those observed at 1 week. Thus, blood AA (1.9 fold) and total omega-6 (1.6 fold) proportions were lower (Figure 4.3.C) and ALA (6.3 fold), EPA (4.7 fold), DPA (2.4 fold) and total omega-3 PUFA (3-fold) proportions (Figure 4.3.D) were higher in offspring of dams in the low LA group compared to high LA groups, irrespective of maternal dietary fat content ($P<0.001$). Proportions of LA were higher in offspring of dams fed the 36%

fat diets compared to those fed 18% fat diets in the high LA group only (P<0.05; Figure 4.3.C), while EPA and DPA proportions were lower in the 36% compared to the 18% fat diet groups, independent of the dietary LA:ALA ratio (P<0.001; Figure 4.3.D). Unlike findings at 1 week of age, the DHA levels in 2 week old offspring of dams consuming a 36% fat diet were lower (P<0.05) when compared to 18% fat groups, irrespective of maternal dietary fatty acid ratio. As at 1 week, SFA proportions were lower (1.2-fold) in offspring of dams fed a 36% fat diet, independent of the LA:ALA ratio (P<0.001). MUFA proportions were 1.2 fold higher in offspring of dams fed the low LA diets, and 1.2 fold higher in offspring of dams who consumed a 36% fat vs 18% diet (P<0.001; Figure 4.3.C).

4.4.6 Offspring organ weight and liver composition

At 1 week of age, heart weight relative to bodyweight was higher in female offspring of dams receiving a 36% fat diet compared to the 18% fat diet, independent of the dietary LA:ALA ratio (P<0.05). There were no differences in the relative weight of lung or kidney at 1 week of age and no differences in the relative weight of the heart, lung, liver, gonadal or retroperitoneal fat pads in the offspring at 2 weeks of age between treatment groups in either male or female offspring (Table 4.3).

Liver weight at 1 week appeared to be influenced by the LA:ALA ratio of the diet to a greater extent than total fat level, at least in males. Thus, male offspring of dams consuming the low LA diets had smaller livers compared to offspring of dams receiving a high LA diet (P<0.01), irrespective of total dietary fat content. The glycogen content of the livers was also lower in male offspring of dams consuming the low LA diet at 1 week (P<0.05). No effect of maternal diet on offspring liver protein or DNA concentration was observed (Table 4.4). These differences were not present in females at 1 week of age and no differences in glycogen content were observed at two weeks of age in male offspring. DNA concentration in females at two weeks of age was marginally increased (1.1-fold) in offspring exposed to a 36% fat diet, irrespective of maternal dietary fatty acid ratio (P<0.05).

4.4.7 Hepatic gene expression

At 1 week of age, hepatic *Fasn* expression was influenced by maternal dietary intervention in a sex specific manner. Thus, in males, *Fasn* expression was higher in

offspring of dams consuming a 36% fat diet irrespective of maternal LA:ALA ratio (P<0.05). In female offspring, however, *Fasn* expression was higher in offspring of dams consuming a low LA diet, independent of dietary fat content (P<0.05). Hepatic *Lpl* mRNA expression in male offspring at 1 week of age was also influenced by maternal dietary fat content, with higher expression in offspring of dams consuming a 36% fat diet vs 18% fat diet (P<0.05). In female offspring, hepatic *Srebf1* expression, similar to that of *Fasn*, was higher in offspring of dams consuming a low LA diet at 1 and 2 weeks of age (P<0.01). Female hepatic expression of *Pparg* was lower in offspring of dams consuming a low LA diet at 2 weeks of age (P<0.05). There were no differences in the expression of *Fasn* or *Lpl* in female offspring, or expression of any hepatic genes in male offspring at this time point (Table 4.3).

4.5 Discussion

This study has demonstrated that altering the fat content and/or LA:ALA ratio of the maternal diet during pregnancy and lactation resulted in significant alteration in the circulating fatty acid profile of dams in the absence of any significant effects on maternal bodyweight or body composition. Exposure to a 36% fat diet during gestation and lactation was, however, associated with lower offspring bodyweight from birth, which persisted to 2 weeks of age. This suggests that increased dietary fat intake during pregnancy and lactation can compromise growth of the progeny, irrespective of the type of fat consumed. In addition, alterations in the fat content and/or composition of the maternal diet had transient effects on offspring body composition and hepatic gene expression, effects which were also sex-specific.

Maternal fatty acid profiles after 4 weeks on the experimental diets largely reflected dietary composition, confirming that the dietary intervention had the desired effect on maternal circulating fatty acid composition. These changes persisted after a further 6 weeks of exposure to the diets and, as expected, the dietary LA:ALA ratio had a greater impact on the maternal blood omega-6 and omega-3 status than total dietary fat content. Consistent with previous studies (Tu *et al.*, 2010, Blank *et al.*, 2002, Brenna *et al.*, 2009, Draycott *et al.*, 2019), decreasing the dietary LA:ALA ratio resulted in substantial increases in relative maternal ALA and EPA levels but only a very modest increase in DHA proportions after a 4-week exposure, and no difference compared to the higher LA:ALA ratio after 10 weeks. Interestingly, and independent of dietary

LA:ALA ratio, dams appeared to be more efficient at converting DPA to DHA when total dietary fat load was higher. One possibility could be that this is simply a result of the higher amount of substrate (i.e. ALA) available for conversion to the longer chain derivatives such as DPA and DHA in diets containing higher total fat levels. This effect did not, however, persist after a further 6 weeks of dietary exposure, at which point EPA and DPA were lower in dams consuming a low LA 36% fat diet compared to a low LA 18%. This may be a result of saturation of the PUFA metabolic pathway when total fat, and therefore PUFA, levels were higher (Tu *et al.*, 2013, Gibson *et al.*, 2013). This apparent decrease in capacity to convert ALA through to EPA and DHA during consumption of a 36% fat diet coincides with the decreased protein intake observed in these groups. It is possible that the lower consumption of protein in rats fed on the 36% fat diets may have contributed to reduced conversion of ALA, since previous studies have shown reduced desaturase, particularly Δ6-desaturase, expression in the mammary gland (Bautista *et al.*, 2013) and liver (Mercuri *et al.*, 1979a) of rats exposed to a low protein diet. Maternal whole blood MUFA proportions appeared to be influenced by dietary LA:ALA ratio, however, this is most likely a result of the slightly higher MUFA content of the low LA diets.

Offspring fatty acid profiles at 1 and 2 weeks of age largely reflected maternal profiles with maternal dietary LA:ALA ratio exhibiting the strongest effect on offspring circulating fatty acid proportions. However, the total fat content of the maternal diet appeared to have a greater influence on the blood fatty acid composition of the offspring as opposed to that observed in the dams. Of particular interest was the finding that the proportion of both EPA and DPA in offspring at 1 week of age were higher in the low LA (18% fat) vs the low LA (36% fat) group, and that this effect persisted at 2 weeks of age despite ALA levels being increased in the low LA (36% fat) group at this time point. DHA was not different between groups at 1 week of age but was lower in offspring exposed to a 36% fat diet at 2 weeks of age. As with the maternal fatty acid profiles, this again may be a result of saturation of the PUFA metabolic pathway at higher total PUFA intakes, and is in line with findings from numerous studies, both human and animal, that indicate that simply increasing the quantity of substrate, i.e. ALA, is not an effective strategy for increasing concentrations of its long-chain derivatives, in particular DHA (Mantzioris *et al.*, 1994, Blank *et al.*, 2002, Brenna *et al.*, 2009, Chan *et al.*, 1993).

The total dietary fat content of the maternal diet also had an influence on the proportion of SFA in the offspring, such that offspring of dams consuming 36% fat diets exhibited lower SFA proportions than offspring of dams consuming the 18% fat diets. Unlike the fetus, where fatty acid composition is largely related to maternal dietary intake, during suckling, offspring fatty acid composition is largely determined by the composition of the milk, which may not fully reflect maternal fatty acid intakes. In a study by Mohammad *et al.* (2014), for example, women consuming diets with a higher total fat content (55%en vs. 25%en) exhibited reduced SFA concentrations (C6:0-C14:0) in breast milk but not in maternal plasma. While milk composition was not assessed in the current study, this raises the possibility that SFA content of the milk may have been lower in those dams consuming the 36% fat diets, which could in turn explain the lower SFA status of the offspring. Alternatively, it may be that increasing the fat content of the diets resulted in an increased conversion of SFA to MUFA, since high-fat feeding has been associated with increased expression of the enzyme responsible for conversion of SFA to MUFA, stearoyl-CoA desaturase 1 (SCD-1) (Hu *et al.*, 2004) and could therefore be the reason for the observed effect of fat content on offspring MUFA levels in this study. It is important to note, however, that circulating fatty acid profiles are a product of both dietary fatty acid intake as well as tissue fatty acid production and release. Whilst the collection of blood samples from animals in the fed state suggests that the dietary fraction of fatty acids would provide a greater contribution to the fatty acid profile of both dams and offspring, the influence of hepatic synthesis of fatty acids should not be overlooked as a contributor to the observed differences.

Despite significant shifts in maternal fatty acid profiles and increased fat content of the 36% fat diets, we saw no differences in maternal bodyweight or fat deposition. This is consistent with our previous study (Draycott *et al.*, 2019) and is likely a result of the reduced feed intakes of the dams to compensate for the increased energy density of the higher fat diets, a phenomenon consistently seen with dietary intervention trials using rodents (Keesey and Hirvonen, 1997). Despite the lack of an effect on maternal weight gain and fat deposition, bodyweight was reduced in offspring of dams receiving a 36% fat diet, irrespective of maternal dietary LA:ALA ratio. This phenotype was consistent across sexes and persisted from birth to 2 weeks of age. Variable results have been reported in this regard with some studies reporting no effects (Khan *et al.*,

2002, Khan *et al.*, 2005, Dyrskog *et al.*, 2005) or increased weight (Samuelsson *et al.*, 2008). This finding was, however, consistent with many other studies that reported decreased fetal (Taylor *et al.*, 2003, Mark *et al.*, 2011), birth (Howie *et al.*, 2009) and weaning weight (Cerf *et al.*, 2006) in offspring of dams exposed to a 36% fat diet during gestation and lactation periods. The differential effects of different high-fat diets on offspring growth is likely due to differences in composition of the diet as well as periods of exposure between studies (Ainge *et al.*, 2011). In those studies that have reported lower offspring weights in offspring fed a high-fat diet, lower protein intakes in dams consuming a high-fat diet have been cited as a likely contributing factor. Further to this, protein restricted diets have been associated with impaired mammary gland development (Bautista *et al.*, 2013, Moretto *et al.*, 2011) leading to impaired milk synthesis (Moretto *et al.*, 2011), and this may also have contributed to reduced offspring growth observed during the suckling period. It is important to note however, that the reduction in protein intake in dams consuming a 36% fat diet in the current study were more modest (10-25%) than those typically used in low-protein diet studies (~50% reduction) (Langley and Jackson, 1994, Langley-Evans and Nwagwu, 1998, Ozanne *et al.*, 1998, Malandro *et al.*, 1996).

The lower *Fasn* expression in the liver and adipose tissue of dams exposed to a 36% fat diet is consistent with the established role of this enzyme in suppressing lipogenesis in times of energy excess (Geelen *et al.*, 2001). Surprisingly, this change did not appear to be mediated through changes in maternal *Srebf1* mRNA expression, a known regulator of *Fasn* expression (Griffin and Sul, 2004). It is important to note that since only mRNA expression was measured, we cannot comment on any differences in protein expression or activity of this transcription factor although mRNA and protein levels have been shown to be closely correlated (Erhuma *et al.*, 2007b). Following this up at the protein level is a major priority for future study. In the offspring, however, hepatic *Fasn* expression was not downregulated by exposure to a maternal 36% fat diet but was actually higher in male offspring of dams consuming the 36% fat compared to the 18% fat diets at 1 week of age and was accompanied by an increase in *Lpl* expression. In female offspring, however, hepatic *Fasn* and *Sbrepf1* expression at 1 week were influenced by maternal dietary fatty acid ratio, rather than total fat content, with both genes upregulated in offspring of dams fed the low LA diets. In both cases, the upregulation of *Sbref1*, *Fasn* and *Lpl* genes would be expected to be

associated with an upregulation of both lipogenesis and fatty acid uptake. It is worth mentioning that differences in hepatic expression of lipogenic genes in male offspring were consistently associated with maternal dietary fat content whereas differences in female hepatic expression were consistently associated with maternal dietary fatty acid ratio. This suggests that female offspring are more sensitive to changes in the types of maternal dietary fat whereas male offspring are more sensitive to gross maternal fat consumption. Sex specific effects associated with the programming of disease hypothesis have been frequently reported (Gabory *et al.*, 2013). The mechanism by which sex influences these effects, however, remains to be elucidated within a larger perspective, as well as within the context of this study.

We found no evidence that these alterations in hepatic gene expression translated to increases in liver weight, however whether there was any effect on hepatic fat content remains to be determined. In both male and female offspring, relative liver weight was increased in offspring of dams fed the high LA diet. In an aim to further elucidate the source of this increased weight, we measured liver DNA, protein and glycogen composition. Similar to liver weight, glycogen levels were increased in offspring of dams fed the high LA diets. This increase in glycogen, however, was not sufficient enough to completely account for the differences observed in liver weight but may be a contributing factor. Consideration of DNA and protein content of the tissue did not indicate significant changes to cell size or number. More detailed analysis is required to further elucidate the mechanism by which high maternal dietary omega-6 may impact upon offspring liver physiology.

The majority of the hepatic mRNA expression differences, as well as gross differences in liver weight and composition, appeared to be transient and were no longer present at 2 weeks of age. A notable exception was the lower expression of *Srebf1* mRNA and higher expression of *Pparg* in females of dams exposed to a high LA diet compared to the low LA diet, with a similar trend observed in males. Although found in relatively low concentrations in the liver, activation of *Pparg* has been shown to increase hepatic lipid storage and is elevated in models of hepatic steatosis (Inoue *et al.*, 2005). As such, decreased *Pparg* expression can alleviate some of the symptoms of hepatic steatosis leading to a reduced liver weight in conjunction with a reduction in hepatic triglyceride content (Gavrilova *et al.*, 2003). Thus, our finding that female offspring

of dams exposed to a high LA diet tended towards to have an increased liver weight at one week of age followed by increased hepatic *Pparg* expression at two weeks of age may suggest that the increase in *Pparg* expression is a potential response to the increased liver growth observed a week earlier. Alternatively, parallels may be drawn to the effect of low protein diets where fluctuations between an increased and decreased lipogenic capacity, chiefly mediated by altered *Srebf1* expression, occur in early life only to settle into a pattern of upregulated lipogenesis at a later life stage (Erhuma *et al.*, 2007b). Further studies would be needed to directly evaluate this hypothesis.

In conclusion, we have demonstrated that exposure to a 36% fat diet during gestation and lactation is associated with persistent growth restriction in both male and female offspring irrespective of maternal dietary fatty acid composition. Growth restriction has been associated with a plethora of metabolic disturbances later in life (Barker, 1995, Phillips *et al.*, 1994, Holemans *et al.*, 2003) and transient alterations in gene expression have been suggested as a mechanism for programming changes in metabolic processes within tissues as well as the morphology of the tissues themselves (Langley-Evans, 2015) . In this study, offspring are still exposed to the experimental diets via the dams milk, and further studies in offspring at older ages are required to assess whether the changes in growth, hepatic gene expression and liver weights in the current study are associated with phenotypic changes that persist once offspring are no longer exposed directly to the altered diet composition. In addition, analysis of lipogenic pathway and adipokine targets at the protein level, as well as whole transcriptome analysis, may yield useful information about their regulation and the extent to which these experimental diets programme other metabolid and regulatory pathways in the liver. Finally, the longevity of these perturbations into later life, especially when presented with secondary metabolic challenges such as aging, prolonged high-fat feeding or in the case of female offspring, pregnancy, remains to be elucidated.

4.6 Tables and Figures

Table 4.1 Maternal organ weights and gene expression

	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Bodyweight (g)	308.75 ± 9.82	288.73 ± 14.95	303.29 ± 11.11	302.23 ± 7.85
Heart (% BW)	0.35 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.35 ± 0.01
Lungs (% BW)[a]	0.45 ± 0.02	0.51 ± 0.04	0.42 ± 0.02	0.48 ± 0.02
Kidney (% BW)	0.78 ± 0.02	0.83 ± 0.03	0.82 ± 0.02	0.79 ± 0.02
Liver (% BW)	5.01 ± 0.10	4.80 ± 0.28	5.28 ± 0.10	4.88 ± 0.09
Brain (% BW)	0.59 ± 0.02	0.59 ± 0.02	0.58 ± 0.02	0.59 ± 0.02
Gonadal Fat (% BW)	1.88 ± 0.35	2.02 ± 0.39	1.65 ± 0.19	1.61 ± 0.11
Retroperitoneal Fat (% BW)	0.76 ± 0.13	0.76 ± 0.13	0.76 ± 0.08	0.85 ± 0.15
Liver mRNA Expression				
Fasn[a*]	20.98 ± 6.17	7.03 ± 1.26	25.08 ± 8.12	9.45 ± 1.63
Lpl	0.20 ± 0.04	0.19 ± 0.04	0.19 ± 0.04	0.13 ± 0.01
Pparg	0.63 ± 0.22	0.78 ± 0.18	0.41 ± 0.09	0.70 ± 0.16
Srebf1	3.52 ± 0.91	2.56 ± 0.64	7.85 ± 2.57	3.39 ± 0.61
Gonadal Fat mRNA Expression				
Fasn[a*]	1.29 ± 0.64	0.18 ± 0.05	2.50 ± 1.16	0.37 ± 0.14
Lpl	0.90 ± 0.23	0.87 ± 0.06	1.56 ± 0.41	1.48 ± 0.46
Ppparg	0.91 ± 0.23	1.22 ± 0.20	1.12 ± 0.13	1.16 ± 0.18
Srebf1	1.80 ± 0.48	1.56 ± 0.31	3.43 ± 1.16	2.21 ± 0.62
Lep	0.49 ± 0.08	1.00 ± 0.29	1.10 ± 0.31	1.38 ± 0.25

All values are mean ± SEM and n=6-9 per dietary group. The effect of dietary fatty acid ratio and dietary fat content were assessed using a two-way ANOVA. [a] indicates a significant effect of dietary fat content ($P<0.05$, *$P<0.01$). Although not statistically significant there was some evidence that maternal *Srebf1* expression was influenced by the LA ($P=0.08$) and fat content ($P=0.06$) of the diet.

Table 4.2 Birth outcomes

	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
n	6	8	7	9
Litter Size	12.83 ± 1.19	13.00 ± 1.21	13.14 ± 0.40	13.33 ± 1.08
Sex Ratio (male/female)	1.01 ± 0.23	0.97 ± 0.24	1.27 ± 0.28	1.13 ± 0.26
Male Birthweight (g)[a]	6.19 ± 0.53	5.19 ± 0.18	5.66 ± 0.14	5.36 ± 0.11
Female Birthweight (g)[a]	5.60 ± 0.37	4.85 ± 0.21	5.26 ± 0.14	5.07 ± 0.12

All values are mean ± SEM. The effect of dietary fatty acid ratio and dietary fat content was assessed using a two-way ANOVA. [a] indicates a significant effect of maternal dietary fat content ($P<0.05$).

Table 4.3 Offspring organ weights and hepatic gene expression

	Male				Female			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**	**High LA (18% Fat)**	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
1 Week Offspring								
Bodyweight (g)	17.52 ± 1.22[a]	12.85 ± 1.16[b]	16.61 ± 0.41[a]	14.20 ± 0.63[b]	15.79 ± 1.11[a]	12.44 ± 1.17[b]	15.66 ± 0.66[a]	13.40 ± 0.56[b]
Heart (% BW)	0.59 ± 0.07	0.67 ± 0.06	0.58 ± 0.04	0.64 ± 0.02	0.56 ± 0.02[a]	0.70 ± 0.06[b]	0.57 ± 0.06[a]	0.69 ± 0.04[b]
Lungs (% BW)	1.87 ± 0.05	1.73 ± 0.04	1.89 ± 0.05	1.90 ± 0.06	1.96 ± 0.11	1.92 ± 0.05	1.88 ± 0.12	1.93 ± 0.05
Kidney (%BW)	1.27 ± 0.08	1.34 ± 0.05	1.19 ± 0.09	1.22 ± 0.02	1.25 ± 0.04	1.38 ± 0.06	1.21 ± 0.10	1.26 ± 0.03
Liver (% BW)	3.17 ± 0.16[a]	3.39 ± 0.13[a]	2.81 ± 0.12[b]	2.89 ± 0.09[b]	3.18 ± 0.10	3.20 ± 0.27	2.96 ± 0.13	2.99 ± 0.05
Liver *Fasn*	0.21 ± 0.08[a]	0.24 ± 0.05[b]	0.18 ± 0.02[a]	0.38 ± 0.04[b]	0.15 ± 0.02[a]	0.22 ± 0.03[a]	0.32 ± 0.06[b]	0.35 ± 0.08[b]
Liver *Lpl*	1.09 ± 0.38[a]	1.26 ± 0.25[b]	0.76 ± 0.15[a]	2.01 ± 0.38[b]	1.26 ± 0.24	1.37 ± 0.46	1.59 ± 0.28	1.81 ± 0.35
Liver *Pparg*	0.40 ± 0.16	0.30 ± 0.07	0.46 ± 0.14	0.38 ± 0.08	0.51 ± 0.11	0.52 ± 0.13	0.62 ± 0.16	0.41 ±0 .06
Liver *Srebpf1*	0.63 ± 0.16	0.56 ± 0.09	0.51 ± 0.10	0.74 ± 0.10	0.44 ± 0.06[a]	0.44 ± 0.05[a]	0.64 ± 0.11[b]	0.80 ± 0.12[b]
2 Week Offspring								
Bodyweight (g)	39.76 ± 1.67[a]	31.78 ± 2.17[b]	39.89 ± 0.59[a]	31.56 ± 1.49[b]	37.77 ± 1.55[a]	31.70 ± 2.05[b]	38.49 ± 0.93[a]	30.75 ± 1.29[b]
Heart (% BW)	0.60 ± 0.01	0.60 ± 0.02	0.61 ± 0.03	0.63 ± 0.01	0.67 ± 0.06	0.67 ± 0.01	0.65 ± 0.03	0.61 ± 0.02
Lungs (% BW)	1.33 ± 0.20	1.26 ± 0.05	1.25 ± 0.07	1.42 ± 0.07	1.28 ± 0.07	1.32 ± 0.05	1.26 ± 0.08	1.32 ± 0.06
Kidney (%BW)	1.05 ± 0.02	1.02 ± 0.03	1.06 ± 0.02	1.00 ± 0.03	1.17 ± 0.04	1.15 ± 0.04	1.14 ± 0.01	1.05 ± 0.02
Gonadal Fat (%BW)	0.22 ± 0.06	0.18 ± 0.02	0.19 ± 0.02	0.18 ± 0.01	0.24 ± 0.02	0.21 ± 0.02	0.23 ± 0.02	0.24 ± 0.03
Retroperitoneal Fat (%BW)	0.36 ± 0.01	0.41 ± 0.04	0.41 ± 0.02	0.39 ± 0.02	0.33 ± 0.03	0.27 ± 0.02	0.29 ± 0.03	0.27 ± 0.01
Liver (% BW)	3.01 ± 0.06	3.08 ± 0.14	3.11 ± 0.02	3.03 ± 0.02	3.18 ± 0.09	3.15 ± 0.09	3.23 ± 0.05	3.01 ± 0.10
Liver *Fasn*	0.17 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.19 ± 0.02	0.20 ± 0.03	0.22 ± 0.03	0.24 ± 0.03
Liver *Lpl*	1.70 ± 0.25[a]	1.81 ± 0.29[b]	1.60 ± 0.13[a]	2.44 ± 0.23[b]	1.25 ± 0.16	1.89 ± 0.16	2.01 ± 0.29	1.81 ± 0.08
Liver *Pparg*	0.56 ± 0.17	0.66 ± 0.10	0.48 ± 0.10	0.42 ± 0.07	0.79 ± 0.25[a]	0.58 ± 0.07[a]	0.31 ± 0.06[b]	0.43 ± 0.07[b]
Liver *Srebf1*	0.74 ± 0.02	0.71 ± 0.08	0.83 ± 0.06	0.80 ± 0.05	0.68 ± 0.07[a]	0.68 ± 0.05[a]	0.83 ± 0.06[b]	0.95 ± 0.10[b]

All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors. Different superscripts denote values which are significantly different ($P<0.05$). n=4-9 per dietary group. All comparisons are made within sex group.

Table 4.4 Offspring liver composition

Experimental Group	Male				Female			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**	**High LA (18% Fat)**	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
1 Week Offspring								
Liver DNA (µg/mg tissue)	0.48 ± 0.06	0.54 ± 0.04	0.56 ± 0.06	0.52 ± 0.03	0.51 ± 0.03	0.51 ± 0.04	0.50 ± 0.04	0.52 ± 0.02
Liver Protein (mg/g tissue)	119.2 ± 12.8	137.7 ± 8.9	135.6 ± 5.2	129.8 ± 4.9	123.8 ± 4.5	138.8 ± 8.3	128.6 ± 3.3	129.5 ± 5.2
Liver Glycogen (µg/mg tissue)	12.71 ± 0.70[a]	11.26 ± 1.86[a]	9.72 ± 1.32[b]	8.64 ± 0.76[b]	9.70 ± 0.89	7.73 ± 0.88	9.00 ± 1.43	11.27 ± 1.80
2 Week Offspring								
Liver DNA (µg/mg tissue)	0.59 ± 0.03	0.53 ± 0.05	0.56 ± 0.04	0.51 ± 0.03	0.52 ± 0.02[a]	0.61 ± 0.05[b]	0.52 ± 0.03[a]	0.57 ± 0.01[b]
Liver Protein (mg/g tissue)	115.1 ± 3.6	129.9 ± 13.5	130.2 ± 10.0	117.9 ± 9.4	117.2 ± 9.7	132.3 ± 9.1	120.7 ± 9.4	120.6 ± 6.5
Liver Glycogen (µg/mg tissue)	9.45 ± 0.61	7.48 ± 0.54	8.35 ± 0.98	9.30 ± 1.75	-	-	-	-

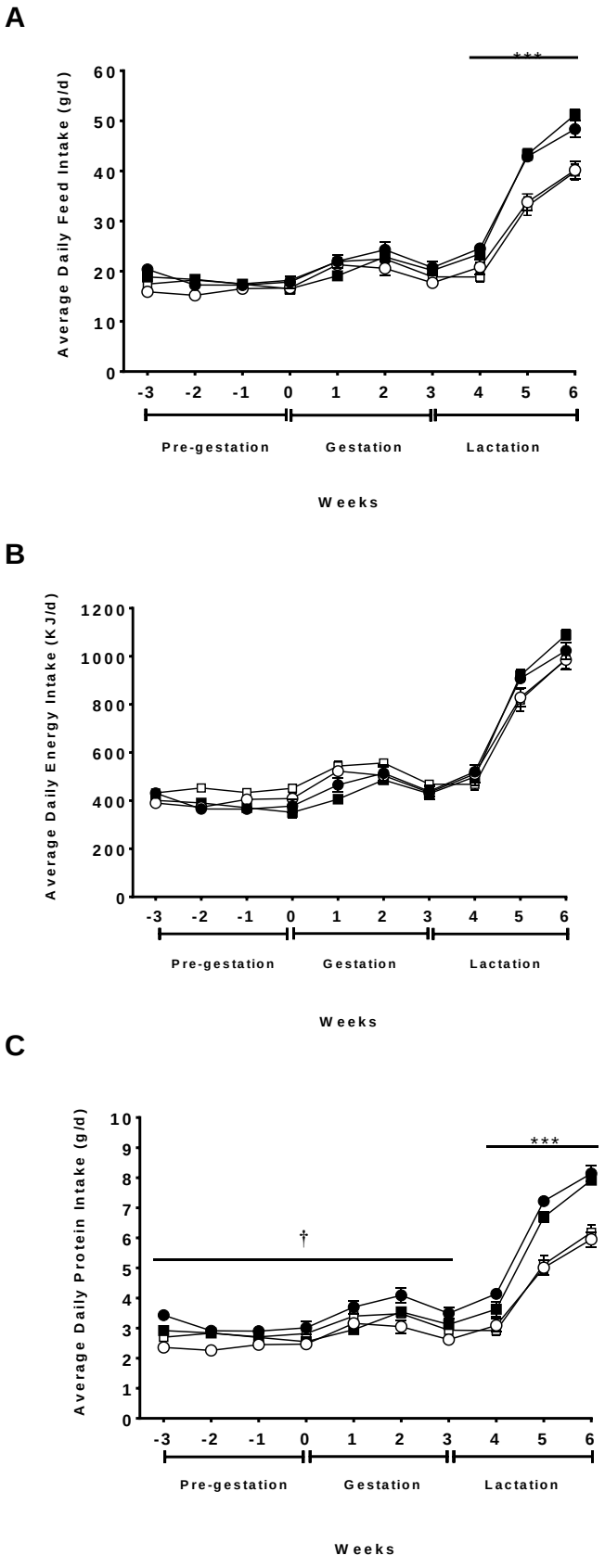
All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors. Different superscripts denote values which are significantly different ($P<0.05$). n=4-9 per dietary group. All comparisons are made within sex group.

Figure 4.1 Maternal average daily (A) feed intake, (B) energy intake and (C) protein intake during pre-feeding, pregnancy and lactation fed on either a high LA (18% fat) diet (closed circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) and a low LA (36% fat) diet (open squares). Values are means ± SEM and n=6-9 per group. The effects of dietary fatty acid ratio and dietary fat content was determined using a two-way repeated measures ANOVA. * indicates a significant effect of dietary fat content (** $P<0.01$, *** $P<0.001$). † indicates a significant interaction between dietary fat content and fatty acid ratio.

Figure 4.2 Maternal whole blood fatty acids profile at (A/B) baseline (C/D) after 4 weeks on experimental diet and (D/E) at the end of lactation (3 weeks post-partum). Values are means ± SEM and n=6-9 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$). † indicates a significant interaction effect ($P<0.05$).

Figure 4.3 Offspring whole blood fatty acids profile at (A/B) one week of age and (C/D) at two weeks of age. Values are means ± SEM and n=11-17 per group. The effects of maternal dietary fatty acid ratio, maternal dietary fat content and sex were determined using a three-way ANOVA. No effect of sex was found for any of the fatty acids measured and so male and female samples were combined for further analysis. * Indicates significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$). † indicates a significant interaction effect ($P<0.05$).

Figure 4.1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40
30
20
10
0
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Pre-gestation
Gestation
Lactation
Weeks
B
Average Daily Energy Intake (KJ/d)
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1000
800
600
400
200
0
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Pre-gestation
Gestation
Lactation
Weeks
C
Average Daily Protein Intake (g/d)
10
9
8
7
6
5
4
3
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Pre-gestation
Gestation
Lactation
Weeks

Figure 4.2

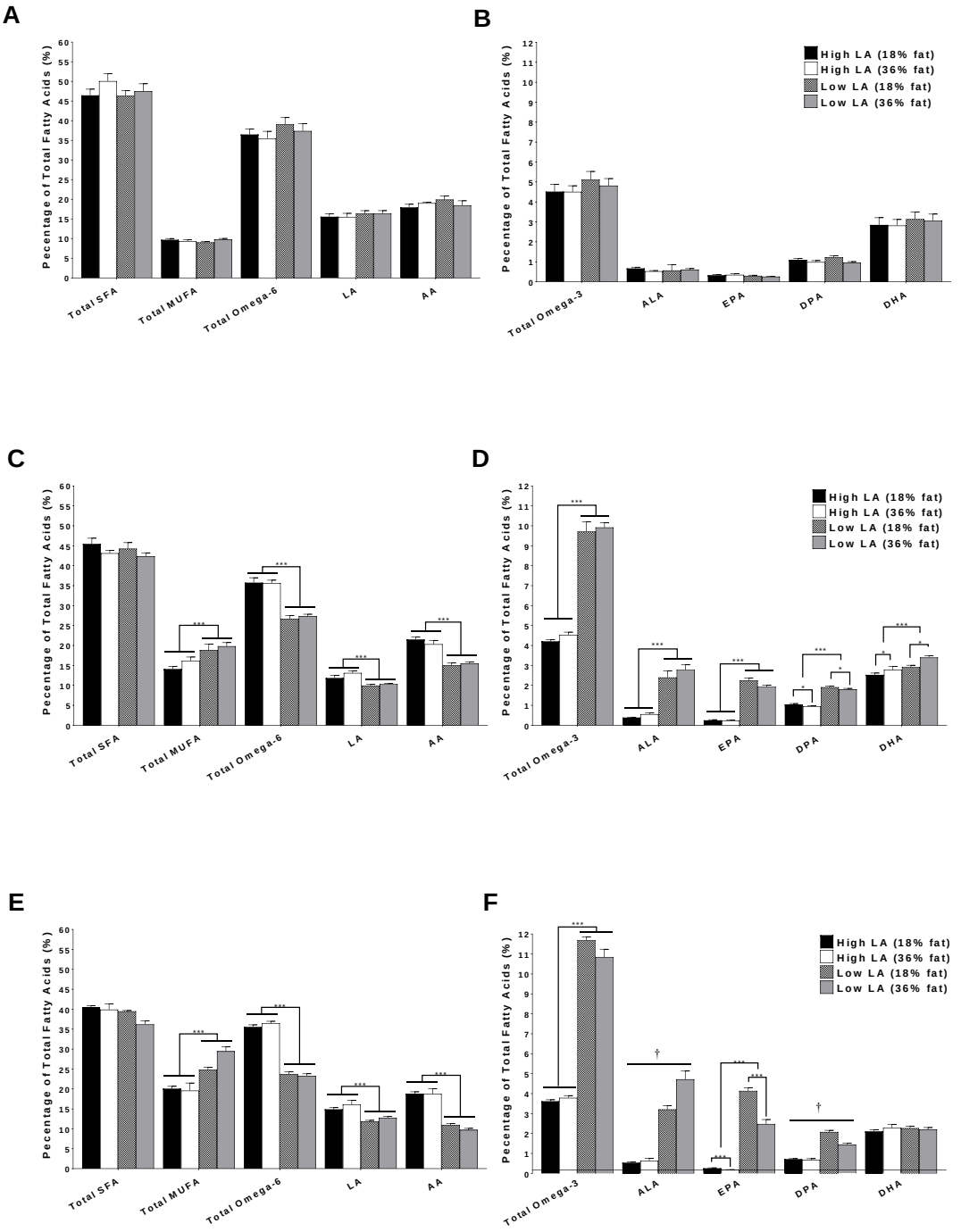
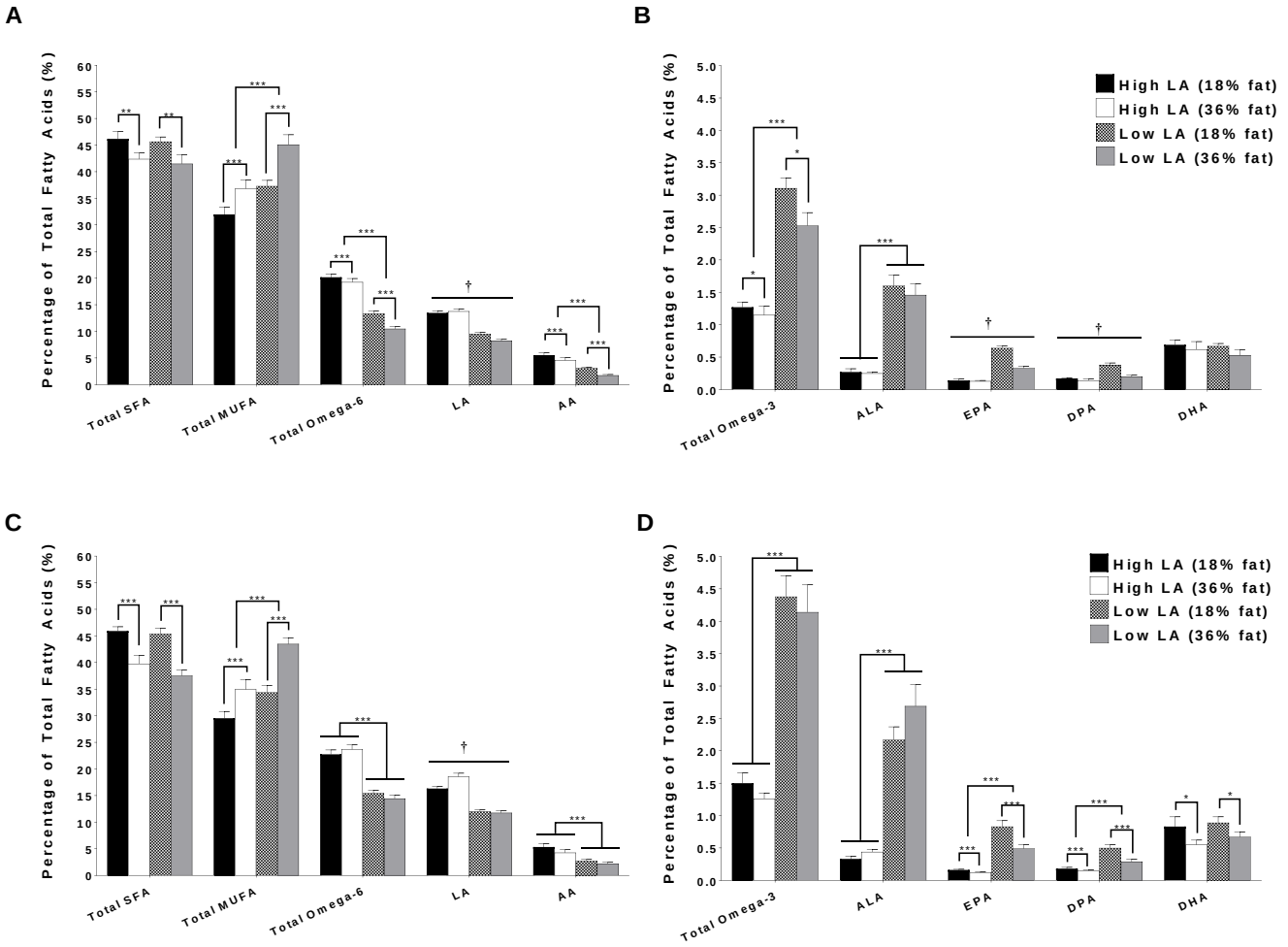

A
B
C
D
E
F
Pecentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)

Figure 4.3


A
B
C
D
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)

5 MANUSCRIPT 3: OMEGA-6:OMEGA-3 FATTY ACID RATIO AND TOTAL FAT CONTENT OF THE MATERNAL DIET ALTERS OFFSPRING GROWTH AND FAT DEPOSITION IN THE RAT

Draycott, SAV, Daniel, Z, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Omega-6:omega-3 fatty acid ratio and total fat content of the maternal diet alters offspring growth and fat deposition in the rat (prepared manuscript).

Author contributions can be found in Appendix C.

5.1 Abstract

Omega-3 long-chain polyunsaturated fatty acids (LCPUFA) have been shown to inhibit lipogenesis and adipogenesis in adult rats. Their possible early life effects on offspring fat deposition, and whether these effects could persist beyond the immediate post-natal period, remains to be established. This study aimed to evaluate the effects of maternal consumption of a linoleic acid (LA) to alpha-linolenic acid (ALA) ratio similar to modern Western diets (9:1) compared to a lower ratio (1:1.5) on offspring adiposity and other health indicators in the post-weaning rat, and to determine any cumulative effects by feeding both diets at two total fat levels (18% vs 36% fat w/w). Female Wistar rats (n=6-9 per group) were fed one of four diets during pregnancy and lactation. Offspring were weaned onto standard laboratory chow. Offspring of dams consuming a high-fat diet, irrespective of maternal dietary LA:ALA ratio, were lighter (male, 27g lighter; female 19g lighter; $P<0.0001$) than offspring of dams fed on an 18% fat diet from 3-8 weeks of age. Offspring exposed to a low LA (18% fat) diet had higher proportions of circulating omega-3 LCPUFA and increased gonadal fat mass at 4 weeks of age ($P<0.05$). Reduced mRNA expression of hepatic ($P<0.01$), gonadal ($P<0.05$) and retroperitoneal ($P<0.05$) *Srebf1* was observed in offspring exposed to a 36% fat diet at 4 weeks of age and in male liver at 8 weeks of age ($P<0.05$). Whilst offspring fat deposition appeared to be sensitive to both maternal dietary LA:ALA ratio and total fat content, offspring growth and lipogenic capacity of tissues was more sensitive to maternal dietary fat content.

5.2 Introduction

Risk of obesity may be partially attributed to the nutritional environment encountered during early life (McMillen and Robinson, 2005). Interventions that target these critical life stages exert a greater preventative effect than those applied later in life (Blake-Lamb *et al.*, 2016). Epidemiological as well as experimental animal studies have shown that exposure to a hypercaloric or high fat diet during early development is associated with increased adiposity in the offspring in later life (Armitage *et al.*, 2005, Hanson and Gluckman, 2014, Wang *et al.*, 2017). Emerging evidence, however, suggests that the type of fat an individual is exposed to during development may also play a key role in determining their future metabolic health. Of increasing interest, due to the significant increase in their consumption over the past 60 years, is the role of dietary omega-6 polyunsaturated fatty acids (PUFA) (Ailhaud *et al.*, 2006, Blasbalg *et al.*, 2011).

The biological effects of omega-6 fatty acids (which have pro-adipogenic and pro-inflammatory properties), as well as the evidence suggesting that increased maternal omega-6 PUFA intake is associated with offspring adiposity (Massiera *et al.*, 2010, Massiera *et al.*, 2003), has led to the hypothesis that a diet high in omega-6 PUFA may be contributing to the increased incidence of obesity (Ailhaud and Guesnet, 2004). Furthermore, due to the opposing anti-inflammatory effects the omega-3 PUFA alpha linolenic acid (ALA) and its derivatives, which are primarily anti-inflammatory in nature, it has been hypothesised that a diet high in these fatty acids may reduce fat deposition (Ruzickova *et al.*, 2004, Hill *et al.*, 2007). The substantial increases in population level intakes of omega-6 PUFA, in particular linoleic acid (LA; precursor to longer omega-6 derivatives) has not coincided with any increases in omega-3 consumption (Blasbalg *et al.*, 2011), resulting in a significant increase in the ratio of omega-6 to omega-3 fatty acids in typical Western diets over this time. Formation of longer chain PUFA, such as arachidonic acid (AA; omega-6), eicosapentaenoic acid (EPA; omega-3) and docosahexaenoic acid (DHA; omega-3), relies on a common set of enzymes utilised by both families of PUFA. As such, competition exists between the two families such that the levels of omega-6 PUFA within the body can directly affect the levels of omega-3 PUFA, therefore, implying that alterations in the ratio of these two families of PUFA, as well as their overall amount, may impact on fat deposition and lipogenesis.

The potential mechanism through which variation in the omega-6:omega-3 ratio in early life may programme metabolic health is unknown. It is possible that early changes in the patterns of expression of key genes involved in lipogenesis within the liver and adipose tissue have a long-term impact on fat deposition and accumulation. These genes include sterol regulatory element-binding protein 1c (*Srebf1*), peroxisome proliferator-activated receptor gamma (*Pparg*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*), and leptin (*Lep*). Previous studies in adult animals have also demonstrated that increased omega-3 PUFA intake can reduce lipid accumulation resulting in an overall reduction in body fat (Hill *et al.*, 2007, Ruzickova *et al.*, 2004, Couet *et al.*, 1997), and that this is mediated through modulation of the expression of *Srebf1* (Xu *et al.*, 1999) and *Pparg* (Forman *et al.*, 1997, Kliewer *et al.*, 1997). There have been few studies, however, investigating whether these anti-lipogenic effects are observed in offspring exposed to a maternal diet that is high in omega-3 fats. Conflicting results have been reported in this regard, with some studies reporting decreased (Ibrahim *et al.*, 2009, Massiera *et al.*, 2003, Wyrwoll *et al.*, 2006) and others reporting increased (Muhlhausler *et al.*, 2011b) offspring adiposity.

The aim of this study was to investigate the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) (Blasbalg *et al.*, 2011), compared to a proposed 'ideal' ratio of ~1:1.5 (Gibson *et al.*, 2011, Lands, 2000) on offspring adiposity and other health indicators. To elucidate any additive effects of altering the maternal dietary LA:ALA ratio, each diet was fed at either 18% fat w/w or at a higher fat content of 36% fat w/w. This paper focusses specifically on the effects of pre- and early post-natal exposure to altered dietary fat content and fatty acid ratio on offspring that have been weaned onto a standard laboratory diet. As such, offspring are no longer directly exposed to the maternal dietary interventions. We hypothesised that exposure to a high LA diet during pregnancy and lactation would lead to increased adiposity in the offspring, marked by increased expression of lipogenic genes, and that this effect may be exacerbated with exposure to a high-fat diet.

5.3 Materials and Methods

5.3.1 Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Virgin female Wistar rats (n=30; 75-100g; Charles River, UK) were maintained as previously described (Draycott *et al.*, 2019). After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status and individuals were then randomly allocated to experimental groups. Animals were maintained on their allocated diet for a four week 'pre-feeding' period, after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Animals were housed in individual cages and remained on their respective diets throughout pregnancy and lactation. All maternal data are reported elsewhere (Draycott *et al*., 2019).

Litters were standardised to 8 pups within 24 hours of birth (4 males and 4 females, where possible). At 1 and 2 weeks of age, one male and one female from each litter were euthanised and tissues collected for analyses, the results of which are published elsewhere (Draycott *et al.*, 2019). At 3 weeks of age, the remaining offspring were weaned and dams were euthanised by CO_2 asphyxiation and cervical dislocation for collection of maternal blood and tissues. Offspring were weaned onto a standard laboratory chow diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK) and pair housed with the remaining same sex littermate. Offspring bodyweight was measured weekly and all animals had blood pressure measured at 4 weeks of age. At this time, one male and one female were euthanised by CO_2 asphyxiation and cervical dislocation. Blood pressure was measured again at 8 weeks of age on all remaining animals after which the experiment ended and all remaining animals were euthanised by CO_2 asphyxiation and cervical dislocation.

5.3.2 Diets

Diets were designed to provide either a high (9:1, high LA) or low (1:1.5, low LA) ratio of LA to ALA. For each level of LA, diets containing either 18% or 36% fat (w/w) were developed. This resulted in four experimental diets; high LA (18% fat),

high LA (36% fat), low LA (18% fat) and low LA (36% fat; n=6-9 per dietary group). The list of ingredients and final fatty acid composition of the four experimental diets are as reported elsewhere (Draycott *et al.*, 2019).

5.3.3 Tail cuff plethysmography

This experiment utilised a non-invasive method for measuring blood pressure validated by Feng *et al.* (2008). A volume pressure recording (VPR) sensor was used to measure tail blood volume to deduce systolic, diastolic and mean arterial blood pressure as well as heart rate. Prior to blood pressure measurements, animals were placed in a heat box set to 30°C for 15 minutes to enhance blood flow to the tail. Animals were then restrained in individual restraint tubes with adjustable nose cone, fitted with the deflated occlusion and VPR cuff (CODA System, Kent Scientific, Torrington, CT) and left to acclimatise to the restraint tube for 10 minutes to minimise the impact of stress before measurements began. After acclimatisation, animals underwent 10 cycles of blood pressure measurements; of these 10 cycles, the first three were disregarded as acclimatisation cycles and an average for each measurement was taken from the remaining seven. Animals were restrained for no longer than 30 minutes and removed if individuals presented with signs of stress.

5.3.4 Blood sample and tissue collection

Blood samples were collected from the offspring at 4 and 8 weeks of age via cardiac puncture and ~30µl was spotted onto PUFAcoat™ dried blood spot (DBS) collection paper (Liu *et al.*, 2014), allowed to dry at room temperature and stored at -20°C for subsequent fatty acid analysis. The remainder of the blood sample was centrifuged at 13000 rpm for 10 minutes at 4°C. The plasma was isolated from the whole blood sample and stored at -80°C until further analysis. Offspring body and organ weights were measured and samples of liver, gonadal fat and retroperitoneal fat were collected at each time point. All tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until determination of gene expression by quantitative reverse transcriptase PCR (q-RTPCR).

5.3.5 Lipid extraction

Total lipids were extracted from liver samples of 4 and 8-week-old offspring. For each sample, ~300mg of crushed, frozen liver was homogenised in 1.6ml of 0.5M Na_2SO_4. The homogenate was decanted into 5.4ml of hexane-isopropanol (3:2, v/v) and 2ml of 0.5M Na_2SO_4 was added. Samples were vortexed and then centrifuged at 3000 rpm for 15 minutes. The supernatant was removed into a fresh tube, dried under nitrogen and the resultant lipid content was weighed. Samples were resuspended in 1ml of hexane and 100µl of resuspended sample was removed into a fresh tube, re-dried under nitrogen and resuspended in 100µl of isopropanol for the determination of cholesterol and triglyceride content. The remaining sample was stored at -20°C for fatty acid analysis.

5.3.6 Determination of circulating and hepatic lipids

Plasma and liver cholesterol and triacylglycerol (TAG) content was determined by a quantitative enzymatic colorimetric assay as per manufacturers protocol (Infinity™ cholesterol and Infinity™ triglyceride reagent; Thermo Scientific UK).

5.3.7 Fatty acid methylation and fatty acid analysis of whole blood and liver samples

Fatty acid composition in maternal and fetal whole blood, and in lipids extracted from liver samples from offspring at 4 weeks of age, was determined by Gas Chromatography (GC) on a Hewlett-Packard 6890 gas chromatograph using methods that have previously been described in detail (Liu *et al.*, 2014, Draycott *et al.*, 2019). Individual fatty acid content was calculated based on peak area and response factors normalised to total fatty acid content and expressed as a percentage of total fatty acids.

5.3.8 Isolation of RNA and cDNA synthesis and reverse transcription quantitative real-time PCR (qRT-PCR)

RNA was isolated from crushed snap-frozen samples of ~25mg of liver using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK). Adipose RNA was extracted, after homogenisation of ~100mg of tissue with MagNA Lyser green beads and instrument (Roche Diagnostics Ltd.), using the RNeasy Mini Kit (QIAGEN Ltd., UK). RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific)

and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Lipogenic pathway and adipokine target genes included; peroxisome proliferator-activated receptor gamma (*Pparg*), sterol regulatory element-binding protein (variant 1c) (*Srebf1*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*) and leptin (*Lep*). Primer sequences for these gene targets have previously been published elsewhere (Draycott *et al.*, 2019). Hepatic expression of delta-5 (*Fads1*; Rn_Fads1_1_SG QuantiTect Primer Assay, Qiagen) and delta-6 (*Fads2*; Rn_Fads2_1_SG QuantiTect Primer Assay, Qiagen) desaturase enzymes were also determined. Cyclophilin A (*PPIA*) and β-actin (*Actb*) were used as housekeeper genes. Adipocyte and hepatic gene expression was quantified using SYBR Green (Roche Diagnostics) in a Light-Cycler 480 (Roche Diagnostics). Samples were analysed against a standard curve of a serially diluted cDNA pool to produce quantitative data and expression was normalised to the housekeeping gene using LightCycler® 480 software (version 1.5.1) as previously described (Rhinn *et al.*, 2008). The expression of the housekeeper genes was not different between treatment groups.

5.3.9 Statistical analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). The effect of maternal dietary fatty acid ratio, maternal dietary fat content and sex on dependent variables was assessed using a three-way ANOVA. Where sex had a main effect on variables but no interaction with maternal dietary factors, data were split for male and female offspring and a two-way ANOVA was used to assess the effect of maternal dietary fat content and fatty acid ratio on male and female offspring separately. Where longitudinal data were analysed, as with bodyweight, the impact of maternal dietary LA:ALA ratio and maternal dietary fat content was analysed using a two-way repeated-measures ANOVA. A value of $P<0.05$ was considered to be statistically significant and dams were used as the unit of analysis.

5.4 Results

5.4.1 Offspring bodyweight, body composition and blood pressure

Offspring bodyweights were measured weekly from birth until the end of the experiment. Figure 5.1 shows bodyweights of offspring from 3-8 weeks of age. Offspring birthweight and bodyweight prior to this are reported elsewhere (Draycott *et al.*, 2019) (see Chapter 4). Offspring of dams consuming a 36% fat diet, irrespective of maternal dietary LA:ALA ratio, were lighter than offspring of dams fed on an 18% fat diet from 3-8 weeks of age in both male (on average 27g lighter) and female (on average 19g lighter) offspring ($P<0.0001$).

Table 5.1 shows the organ and fat depot weights of male and female offspring at 4 and 8 weeks of age. At 4 weeks of age, relative heart weight was 5% higher and relative liver weight was 4% lower in female offspring of dams exposed to a 36% fat diet compared to those exposed to an 18% fat diet, irrespective of maternal dietary LA:ALA ratio ($P<0.05$). Relative liver weight at 4 weeks also tended ($P=0.075$) to be lower in male offspring of dams provided the 36% vs 18% fat diet. A significant interaction between maternal dietary fatty acid ratio and maternal dietary fat content on relative gonadal fat weight was observed for both male and female offspring at 4 weeks of age, manifesting as ~30% lower weight of the gonadal fat depots in the low LA group, but only if exposed to 36% fat in early life. There were no differences in the relative weight of lungs, kidney or retroperitoneal fat pad between experimental groups at 4 weeks of age. Differences in relative organ and fat weights measured in offspring at 4 weeks of age appeared to be transient as no differences were observed at 8 weeks of age for any of these organs or fat depots.

Blood pressure at 4 weeks of age was not influenced by maternal diet. At 8 weeks of age, female offspring exposed to a 36% diet during gestation and lactation had significantly lower systolic (16 mmHg; $P=0.024$) and tended to have lower diastolic (11 mmHg; $P=0.068$) blood pressure than offspring exposed to an 18% fat diet (Table 5.1). Blood pressure in males was not influenced by either LA:ALA ratio or fat content of the maternal diet.

5.4.2 Offspring whole blood and hepatic fatty acid profile

A significant effect of sex was observed for some of the fatty acids measured in whole blood and liver at 4 and 8 weeks of age. No interactions were observed between sex and maternal dietary treatment, so male and female data were split for further analysis. Figure 5.2 shows the fatty acid profile of whole blood in offspring at 4 weeks of age. In male offspring, exposure to a 36% fat diet was associated with increased proportions of saturated fatty acid (SFA; $P<0.05$) and monounsaturated fatty acid (MUFA; $P<0.05$) as well as decreased proportions of LA ($P<0.05$) and AA ($P<0.05$), resulting in lower overall total omega-6 in response to a maternal 36% fat diet. Proportions of MUFA and AA were also influenced by maternal dietary fatty acid ratio such that a low LA diet was associated with increased MUFA ($P<0.01$) and decreased AA ($P<0.01$) levels. A similar pattern was observed for the proportions of SFA, MUFA and omega-6 fatty acids in whole blood of female offspring. In females, a significant interaction was observed for the proportions of long chain omega-3 fatty acids (EPA, $P<0.05$; DPA, $P<0.01$ and DHA; $P<0.05$). Interestingly, female offspring exposed to a low LA (18% fat) diet had higher proportions of these fatty acids and as a result, higher total omega-3 proportions. Similar patterns were observed in male offspring, however, only a significant main effect of maternal dietary LA:ALA ratio was observed.

The elevated omega-3 proportions in offspring of dams exposed to a low LA (18% fat) diet at 4 weeks of age, prompted investigation into the liver fatty acid profile at this time point (Figure 5.3). Interestingly, the composition of fatty acids in the liver did not completely reflect that of the whole blood, and were only influenced by maternal dietary fatty acid ratio. In male offspring, exposure to a low LA diet during pregnancy and lactation was associated with lower proportions of total omega-6, LA and AA and higher proportions of total omega-3, ALA, EPA, DHA and total SFA (Figure 5.3.A). Similar observations were made for the fatty acid composition of the liver in female offspring at this time point. A key difference, however, was that maternal diet appeared to have no effect on total SFA in the female offspring (Figure 5.3.B).

At 8 weeks of age, whole blood fatty acid profile was reassessed (Figure 5.4). In male offspring, there were no longer any differences in proportions of SFA, MUFA, total omega-6, LA, AA or ALA between experimental groups. Total omega-3 ($P<0.001$), EPA ($P<0.01$), DPA ($P<0.05$) and DHA ($P=0.052$) proportions all remained elevated

in male offspring of dams exposed to a low LA diet during pregnancy and lactation. Similar observations were made for the fatty acid composition of female whole blood at 8 weeks of age. Proportions of AA, and consequently levels of total omega-6, were, however, higher in female offspring exposed to a high LA diet, irrespective of maternal dietary fat content. In both male and female whole blood, DPA proportions appeared to be influenced by maternal dietary fat content such that a 36% fat diet was associated with lower proportions of this fatty acid. This was significant in female offspring ($P<0.05$) and tended towards significance in male offspring ($P=0.057$). Unlike in male offspring, DHA proportions in female offspring whole blood at 8 weeks of age were not associated with maternal dietary intake.

5.4.3 Circulating and hepatic lipid profile

At 4 weeks of age, male offspring exposed to a 36% fat diet had lower circulating plasma TAG concentrations ($P=0.01$) and reduced liver cholesterol concentrations ($P<0.05$) when compared to offspring exposed to an 18% fat diet (Table 5.2). In the female liver, however, TAG concentrations were affected by maternal dietary ratio such that female offspring exposed to a low LA diet had lower concentrations of liver TAG ($P<0.05$), irrespective of maternal dietary fat level. There was no effect of maternal diet on plasma cholesterol or total liver lipid at 4 weeks of age. By 8 weeks of age, there were no significant differences in any of the variables measured in female offspring. In males, however, there was a significant interaction of maternal dietary fat content and fatty acid ratio such that exposure to a high LA (36% fat) diet resulted in increased circulating cholesterol ($P<0.05$) but reduced liver TAG concentrations in 8 week old male offspring (Table 5.2).

5.4.4 Gene expression

An interaction between maternal dietary fatty acid ratio and fat content on hepatic expression of *Fads1* and *Fads2* was observed in female offspring (Table 5.2). This resulted in increased expression of both genes in female offspring of dams exposed to a high LA (36% fat) diet suggesting an increased capacity for synthesis of long chain fatty acids in this group. There were no differences in the expression of these genes in male offspring. Expression of key lipogenic genes (*Fasn, Lpl, Pparg, Srebf1 and Lep*) was measured in the liver as well as gonadal and retroperitoneal fat depots (*Lep* was

only measured in the fat depots due to limited hepatic expression). At 4 weeks of age (Table 5.3), a consistent effect of maternal dietary fat content on expression of *Srebf1* mRNA was observed. Offspring of dams exposed to a 36% fat diet had lower expression of hepatic ($P<0.01$), gonadal fat ($P<0.05$; significant in female offspring only) and retroperitoneal fat ($P<0.05$) *Srebf1* mRNA compared to offspring of dams consuming an 18% fat diet. A similar pattern was observed for other genes in the retroperitoneal fat depot, such that offspring of dams exposed to a 36% fat diet exhibited lower mRNA expression of *Fasn* ($P<0.01$; male offspring only), *Lpl* ($P<0.05$; male and female offspring) and *Lep* ($P<0.05$; male offspring only). At 4 weeks of age, hepatic *Lpl* expression was higher in offspring of dams consuming a low LA 36% fat diet compared to other groups in both male and female offspring ($P<0.05$). In female offspring at 4 weeks of age, a significant interaction between maternal dietary fatty acid ratio and maternal dietary fat content was observed in gonadal fat expression of *Fasn* ($P<0.05$) and *Lep* ($P<0.05$) as well as retroperitoneal fat expression of *Pparg* ($P<0.05$) and *Lep* ($P<0.01$). This interaction manifested as increased expression of these genes in offspring exposed to 36% fat with a high LA:ALA ratio, but decreased expression when the diet consisted of a low LA:ALA ratio. As such, offspring exposed to a low LA (18% fat) diet consistently exhibited the highest expression of these genes.

Table 5.4 summarises the mRNA expression at 8 weeks of age. Male offspring exposed to a 36% fat diet during gestation and lactation showed lower hepatic *Srebf1* and gonadal fat *Lep* mRNA expression ($P<0.05$) when compared to an 18% fat diet, irrespective of maternal dietary fatty acid ratio. In females, and other tissues measured in male offspring, the differences in *Srebf1* expression observed at 4 weeks of age appeared to be transient as no differences were observed between groups at 8 weeks of age. *Fasn* mRNA at 8 weeks of age was significantly higher in female offspring of dams consuming a 36% fat diet in both the gonadal ($P<0.05$) and retroperitoneal ($P<0.01$) fat depots, irrespective of maternal dietary ratio.

5.5 Discussion

This study aimed to investigate the effect of an altered maternal dietary LA:ALA ratio, as well as total dietary fat content, on offspring growth, adiposity, lipid profiles and expression of key genes associated with lipogenesis. We have shown that the maternal

dietary LA:ALA ratio is a key driver of the fatty acid profile in offspring whole blood and liver. Additionally, we found that exposure to a high fat diet, irrespective of dietary LA:ALA ratio, was associated with a persistent reduction in offspring bodyweight even after the offspring were weaned onto a standard, nutritionally balanced rodent diet. Differences in adipose tissue weight were determined by maternal dietary LA:ALA ratio as well as total fat content, whilst the expression of key lipogenic genes was predominantly affected by the latter. These data suggest that a maternal diet high in fat can have detrimental effects on offspring growth whilst an interaction between total fat intake and maternal dietary PUFA ratio appears to affect offspring adiposity via alterations in the expression of lipogenic genes.

We have previously shown that exposure to a varying LA:ALA ratio and fat content in the diet influences the circulating fatty acid profile of dams (Draycott *et al.*, 2019) as well as offspring directly exposed to the maternal diet (see Chapter 4). In the present study, we have demonstrated that the circulating and hepatic fatty acid profiles of offspring at 4 weeks of age, as well as the circulating fatty acid profile at 8 weeks of age, are still influenced by maternal dietary factors despite the offspring no longer being directly exposed to dietary interventions. Of particular interest is the elevated proportions of long chain omega-3 PUFA in whole blood samples of offspring exposed to a low LA (18% fat) diet but not in those exposed to a low LA (36% fat) diet. The experimental diet, as well as the chow diet that offspring were weaned onto, only contained the omega-6 and omega-3 precursors, LA and ALA. This implies, therefore, that the increased levels of long-chain omega-3 PUFA (LCPUFA) are due to an increased capacity of these offspring for conversion of ALA to its longer-chain derivatives through elongation and desaturation and/or remnants of preferential transfer of these fatty acids from the mother during pregnancy and/or lactation. We are inclined to believe this is a result of the latter as our previous study indicated a similar fatty acid profile in the dams during the lactation period (see Chapter 4). Further to this, studies in other species have provided no evidence of increased desaturation and elongation capacity of offspring exposed to higher omega-3 levels (Kanakri *et al.*, 2017). However, a combination of both of these factors, as well as the possible influence of fatty acid release from adipose tissue, is conceivable and should not be completely ruled out.

This interesting finding in the offspring whole blood fatty acid profile at 4 weeks of age encouraged investigation into the hepatic fatty acid profile and capacity for long chain PUFA synthesis. Interestingly, and despite evidence of strong correlations between circulating and hepatic liver fatty acid profiles (Tu *et al.*, 2013), we found that the increased omega-3 LCPUFA observed in whole blood of offspring exposed to a low LA (18% fat) diet was not apparent in the liver. Investigation into the desaturation and elongation capacity of the liver in these animals revealed some sex-specific interactions of maternal diet and key genes associated with this pathway. The observation that female offspring exposed to a high LA (36% fat) diet exhibited increased levels of *Fads1* and *Fads2*, does in fact suggest that these individuals may have an increased capacity for LCPUFA synthesis. This did not, however, appear to translate into any physiological differences in the composition of fatty acids in the liver between experimental groups and the mRNA levels measured in this study may not be reflective of protein levels and/or activity of these enzymes. These findings highlight the potential for prolonged biological effects of fatty acids incorporated into phospholipid membranes and/or stored in tissues during gestation and lactation. Further experiments investigating the longevity of changes in offspring fatty acid profile would confirm if there is a programmed effect of increased capacity for LCPUFA synthesis or if this is an artefact of direct exposure to the maternal dietary intervention. Even if transient, the effects of altered fatty acid composition of tissues, restriction of growth and greater adiposity that we have observed are likely to potentiate long-term metabolic consequences.

Offspring of dams exposed to a 36% fat diet exhibited consistently lower bodyweights than offspring exposed to an 18% fat diet during gestation and lactation. Importantly, this effect was apparent from birth (see Chapter 4) and persisted after the offspring had been weaned onto a standard laboratory diet, suggesting a long-term effect of exposure to a maternal high-fat diet that is persistent beyond direct dietary exposure. This is consistent with many studies reporting decreased fetal (Taylor *et al.*, 2003, Mark *et al.*, 2011), birth (Howie *et al.*, 2009) and weaning weight (Cerf *et al.*, 2006) in offspring of dams exposed to a high-fat diet during gestation and lactation periods. Early life growth restriction is often proceeded by a period of "catch-up" growth in which offspring gain weight rapidly and is often associated with increased adiposity (Ong *et al.*, 2000) and increased risk of metabolic disease and hypertension in the

offspring (Morrison *et al.*, 2010). Surprisingly, further to a reduced bodyweight in response to a maternal high-fat diet, female offspring at 8 weeks of age also had reduced blood pressure. This apparent increased sensitivity of female rather than male offspring to maternal dietary treatments has been noted previously (Khan *et al.*, 2002), although, as in a number of other studies (Samuelsson *et al.*, 2008, Guberman *et al.*, 2013), maternal high-fat diets resulted in increased as opposed to decreased blood pressure. It is important to note, however, that many of these studies utilised a maternal diet high in saturated fat and studies using diets high in polyunsaturated fats, as with this study, have shown reductions in blood pressure when compared to a maternal diet high in saturated fats (Langley-Evans *et al.*, 1996a). In support of this, we have previously noted that offspring hypertension associated with low protein feeding during rat pregnancy is modified by other components of the experimental diet, including the source of fat (Langley-Evans, 2000, Langley-Evans, 1996).

An unexpected finding of the study was that offspring exposed to a low LA (18% fat) diet had the highest relative gonadal fat mass at 4 weeks of age, in conjunction with higher *Lep* mRNA expression in the gonadal fat adipose tissue. This was consistent across sexes and conflicted with our hypothesis as well as the evidence linking increased omega-3 intake with reduced fat deposition and accumulation in *in vitro* and rodent models (Massiera *et al.*, 2003, Wyrwoll *et al.*, 2006). This finding was, however, in line with other rodent studies in which the higher omega-3 exposure was restricted to the gestation and lactation periods (Muhlhausler *et al.*, 2011b) and adds to the disparity observed in reports of human (Muhlhausler *et al.*, 2010b) as well as animal studies (Muhlhausler *et al.*, 2011a) investigating the role of increased maternal dietary omega-3 on offspring body composition. The increased gonadal fat weight of the low LA (18% fat) group at 4 weeks of age coincided with increased *Fasn* expression in this tissue, suggesting that the higher gonadal fat deposition may have been driven by an increased capacity for *de novo* lipogenesis in this group. Interestingly, however, this increased *Fasn* expression was only observed in females, raising the possibility of different underlying mechanisms for increased gonadal fat accumulation between males and females which has also been suggested by previous work (Vithayathil *et al.*, 2018).

Interestingly, and despite no effects on relative mass, expression of lipogenic genes in the retroperitoneal fat depot appeared to be more susceptible to maternal dietary effects compared to the gonadal fat depot. In both male and female offspring at 4 weeks of age, there appeared to be a decrease in lipogenic capacity in offspring of dams fed high-fat diets. This apparent reduction in lipogenic capacity may be a compensatory response to mitigate the effects of a maternal high-fat diet and limit excessive fat accumulation in these groups which has been demonstrated in rodent models directly consuming a high-fat diet (Pichon *et al.*, 2006, Ferramosca *et al.*, 2014, Duarte *et al.*, 2014, Reynés *et al.*, 2014). Indications of reduced lipogenic capacity were also apparent in the liver in offspring of dams exposed to a high-fat diet. A significant reduction in hepatic *Srebf1* expression was observed in offspring of dams receiving a 36% fat diet; this was accompanied by proportionally lower liver weights in these groups at 4 weeks of age. In other models of maternal dietary insult, such as the low protein model, studies have shown that early reductions in the lipogenic capacity of tissues, through reduced gene expression, are often followed by an upregulation in lipogenesis between 9 and 18 months of age (Erhuma *et al.*, 2007b), but can occur much earlier if the individual encounters further dietary challenge (Erhuma *et al.*, 2007a). Whilst this study only followed offspring until 8 weeks of age, some indications of this shift in lipogenic capacity were apparent in female offspring at this time point. Female offspring of dams exposed to a 36% fat diet exhibited increased *Fasn* levels suggesting increased *de novo* lipogenesis in these tissues. Long-term studies are required to evaluate these findings further.

In conclusion, we have shown that, despite significant alteration in the ratio of omega-3 and omega-6 fatty acids in offspring of dams fed either a high or low LA diet, offspring growth and lipogenic capacity of adipose tissue are more susceptible to changes in the total fat content of the maternal diet rather than changes in the types of fats consumed. Whilst there appears to be more robust data supporting the beneficial effects of omega-3 fatty acids on mature adipocytes (Hill *et al.*, 2007, Ruzickova *et al.*, 2004, Couet *et al.*, 1997), their biological effects on developing adipose tissue are far less clear. Evidence suggesting beneficial or detrimental effects of the two families of PUFA in the maternal diet on offspring growth and adiposity, have largely been based on *in vitro* studies or animal experiments and recent data have suggested limited reproduction of these results in human trials (Meyer *et al.*, 2019). Further studies are

required to investigate the effects of maternal dietary PUFA on developing tissues but caution should be exercised in the meantime not to extrapolate from data on mature tissues and to highlight the detrimental effects of a maternal high-fat intake regardless of the types of fats consumed.

5.6 Tables and Figures

Table 5.1 Offspring body composition and blood pressure at 4 and 8 weeks of age

Experimental Group	Male				Female			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**	**High LA (18% Fat)**	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
4 Week Offspring								
Heart (% BW)	0.49 ± 0.02	0.53 ± 0.02	0.50 ± 0.02	0.51 ± 0.02	0.52 ± 0.003[a]	0.55 ± 0.002[b]	0.51 ± 0.02[a]	0.53 ± 0.01[b]
Lungs (% BW)	1.05 ± 0.07	1.07 ± 0.10	1.02 ± 0.07	1.29 ± 0.06	1.39 ± 0.09	1.28 ± 0.12	1.20 ± 0.11	1.19 ± 0.08
Kidney (%BW)	1.08 ± 0.03	1.12 ± 0.03	1.10 ± 0.02	1.06 ± 0.01	1.02 ± 0.10	1.11 ± 0.02	1.13 ± 0.04	1.05± 0.02
Liver (% BW)	5.05 ± 0.15	4.92 ± 0.13	5.05 ± 0.12	4.71 ± 0.10	4.98 ± 0.07[a]	4.73 ± 0.08[b]	4.76 ± 0.16[a]	4.60 ± 0.11[b]
Gonadal fat (%BW) *	0.49 ± 0.03	0.52 ± 0.04	0.56 ± 0.03	0.39 ± 0.03	0.64 ± 0.09	0.66 ± 0.05	0.79 ± 0.05	0.54 ± 0.04
Retroperitoneal fat (%BW)	0.57 ± 0.06	0.52 ± 0.04	0.52 ± 0.07	0.47 ± 0.05	0.42 ± 0.06	0.37 ± 0.03	0.42 ± 0.03	0.34 ± 0.03
Systolic BP (mmHg)	86.2 ± 4.0	92.6 ± 4.6	89.0 ± 5.9	91.9 ± 3.8	85.8 ± 7.2	87.4 ± 3.3	91.8 ± 4.1	91.3 ± 5.2
Diastolic BP (mmHg)	65.5 ± 3.6	68.2 ± 3.7	65.4 ± 4.6	68.3 ± 3.3	61.9 ± 7.1	61.1 ± 2.1	68.9 ± 4.1	64.5 ± 2.2
8 Week Offspring								
Brain (%BW)	0.56 ± 0.02	0.57 ± 0.02	0.55 ± 0.02	0.57 ± 0.01	0.80 ± 0.01	0.84 ± 0.02	0.79 ± 0.01	0.79 ± 0.03
Heart (% BW)	0.34 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.39 ± 0.02	0.40 ± 0.01
Lungs (% BW)	0.61 ± 0.06	0.62 ± 0.05	0.61 ± 0.05	0.63 ± 0.04	0.66 ± 0.04	0.68 ± 0.04	0.60 ± 0.02	0.68 ± 0.05
Kidney (%BW)	0.83 ± 0.04	0.83 ± 0.02	0.84 ± 0.02	0.88 ± 0.03	0.88 ± 0.03	0.87 ± 0.03	0.85 ± 0.03	0.81 ± 0.02
Liver (% BW)	4.80 ± 0.08	5.00 ± 0.14	4.96 ± 0.09	5.00 ± 0.08	4.49 ± 0.16	4.63 ± 0.08	4.43 ± 0.10	4.56 ± 0.08
Gonadal fat (%BW)	1.30 ± 0.10	1.25 ± 0.08	1.34 ± 0.08	1.19 ± 0.09	1.38 ± 0.15	1.56 ± 0.07	1.53 ± 0.19	1.57 ± 0.23
Retroperitoneal fat (%BW)	1.22 ± 0.09	1.29 ± 0.11	1.32 ± 0.11	1.16 ± 0.10	0.88 ± 0.10	0.94 ± 0.11	0.94 ± 0.09	0.86 ± 0.08
Systolic BP (mmHg)	116.0 ± 5.0	109.1 ± 5.2	113.4 ± 3.4	105.8 ± 6.6	122.7 ± 10.1[a]	103.2 ± 3.0[b]	120.4 ± 8.2[a]	107.4 ± 5.9[b]
Diastolic BP (mmHg)	82.9 ± 0.2	73.5 ± 5.3	80.3 ± 3.3	74.4 ± 5.7	82.3 ± 5.1	71.8 ± 2.3	86.0 ± 7.1	74.5 ± 6.3

All values are mean ± SEM and organ weights are expressed as a percentage of bodyweight (%BW). A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors, all comparisons are made within sex groups. Different superscripts denote values which are significantly different ($P<0.05$). * indicates a significant interaction effect of maternal dietary fatty acid ratio and maternal fat content on gonadal fat weight in male ($P<0.01$) and female ($P<0.05$) offspring. n=6-9 per dietary group.

Table 5.2 Offspring lipid and liver characteristics at 4 and 8 weeks of age

Experimental Group	Male				Female			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**	**High LA (18% Fat)**	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
4 Week Offspring								
Plasma cholesterol (mmol/L)	2.93 ± 0.13	3.08 ± 0.10	3.08 ± 0.13	2.90 ± 0.15	2.83 ± 0.09	2.90 ± 0.18	2.80 ± 0.14	2.55 ± 0.10
Plasma TAG (mmol/L)	0.89 ± 0.07[a]	0.74 ± 0.03[b]	1.03 ± 0.12[a]	0.75 ± 0.06[b]	0.72 ± 0.06	0.76 ± 0.05	0.83 ± 0.09	0.80 ± 0.09
Liver lipid (mg/g tissue)	30.59 ± 4.37	33.60 ± 3.51	28.55 ± 6.07	33.53 ± 1.28	34.33 ±7.35	36.61 ± 3.25	26.03 ± 3.01	28.57 ± 4.00
Liver cholesterol (mg/g tissue)	1.93 ± 0.20[a]	1.46 ± 0.12[b]	1.78 ± 0.15[a]	1.63 ± 0.11[b]	1.63 ± 0.18	1.58 ± 0.12	1.56 ± 0.15	1.55 ± 0.09
Liver TAG (mg/g tissue)	18.01 ± 1.71	16.40 ± 1.54	16.01 ± 2.64	13.23 ± 1.90	17.05 ± 2.42[a]	15.75 ± 1.37[a]	13.99 ± 1.03[b]	11.56 ± 0.85[b]
Liver *Fads1*[#]	0.99 ± 0.15	1.31 ± 0.21	0.88 ± 0.07	0.97 ± 0.20	1.09 ± 0.06	1.48 ± 0.21	1.02 ± 0.10	0.82 ± 0.08
Liver *Fads2*[#]	1.08 ± 0.13	1.42 ± 0.18	1.27 ± 0.19	1.16 ± 0.16	1.19 ± 0.06	1.54 ± 0.16	1.33 ± 0.19	0.95 ± 0.06
8 Week Offspring								
Plasma cholesterol (mmol/L)*	2.39 ± 0.14	3.47 ± 0.33	2.87 ± 0.14	2.97 ± 0.15	2.30 ± 0.30	2.72 ± 0.21	2.72 ± 0.17	2.64 ± 0.15
Plasma TAG (mmol/L)	1.57 ± 0.18	1.48 ± 0.17	1.42 ± 0.09	1.51 ± 0.11	1.10 ± 0.16	0.92 ± 0.06	1.11 ± 0.13	1.03 ± 0.11
Liver lipid (mg/g tissue)	37.56 ± 3.69	36.18 ± 6.82	28.22 ± 10.63	26.27 ± 3.47	26.81 ± 2.27	32.73 ± 6.80	30.99 ± 5.70	37.27 ± 7.01
Liver cholesterol (mg/g tissue)	1.20 ± 0.06	1.06 ± 0.12	1.11 ± 0.12	0.97 ± 0.10	0.87 ± 0.13	1.16 ± 1.38	0.89 ± 0.17	0.94 ± 0.12
Liver TAG (mg/g tissue)*	19.87 ± 1.80	9.81 ± 1.13	13.68 ± 1.99	12.50 ± 1.87	14.53 ± 1.29	12.15 ± 2.53	11.72 ± 0.89	8.91 ± 1.24

All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors; all comparisons are made within sex group. Different superscripts denote values which are significantly different ($P<0.05$). A significant interaction of maternal dietary fat content and maternal dietary fatty acid is denoted for *males ($P<0.05$) and [#]females ($P<0.05$). n=6-9 per dietary group.

Table 5.3 Offspring lipogenic gene expression in male and female offspring at 4 weeks of age

Experimental Group	Male				Female			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**	**High LA (18% Fat)**	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
4 Week Offspring								
Liver								
Fasn	0.91 ± 0.12	0.93 ± 0.15	1.49 ± 0.42	0.66 ± 0.08	0.97 ± 0.14	1.08 ± 0.10	1.12 ± 0.19	0.90 ± 0.08
Lpl$^{\dagger\#}$	0.12 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	0.18 ± 0.02	0.15 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.18 ± 0.03
Pparg	0.95 ± 0.18	0.91 ± 0.11	0.94 ± 0.17	0.91 ± 0.17	0.96 ± 0.22	0.78 ± 0.12	0.70 ± 0.10	0.75 ± 0.13
Srebf1	0.82 ± 0.13^{a}	0.64 ± 0.02^{b}	0.89 ± 0.11^{a}	0.52 ± 0.07^{b}	0.86 ± 0.10^{a}	0.65 ± 0.07^{b}	0.79 ± 0.10^{a}	0.40 ± 0.05^{b}
Gonadal Fat								
Fasn$^{\#}$	3.23 ± 1.17	2.22 ± 0.52	2.36 ± 0.37	1.17 ± 0.06	0.85 ± 0.16	2.42 ± 0.46	3.08 ± 0.47	2.29 ± 0.59
Lpl	0.90 ± 0.05	0.94 ± 0.13	1.30 ± 0.33	0.80 ± 0.12	1.04 ± 0.30	0.88 ± 0.04	1.47 ± 0.27	1.07 ± 0.17
Pparg	0.84 ± 0.04	0.87 ± 0.08	0.95 ± 0.12	0.86 ± 0.03	0.68 ± 0.14	0.80 ± 0.12	0.81 ± 0.06	0.56 ± 0.09
Srebf1	3.12 ± 0.65	3.05 ± 0.50	3.29 ± 0.51	2.21 ± 0.27	3.15 ± 0.64^{a}	2.23 ± 0.18^{b}	3.68 ± 0.63^{a}	2.57 ± 0.45^{b}
Lep$^{\#}$	0.90 ± 0.22	0.83 ± 0.08	1.01 ± 0.12	0.62 ± 0.12	0.38 ± 0.05	0.72 ± 0.12	1.32 ± 0.25	0.70 ± 0.14
Retroperitoneal Fat								
Fasn	5.19 ± 1.09^{a}	2.35 ± 0.37^{b}	3.01 ± 0.37^{a}	2.19 ± 0.45^{b}	1.86 ± 0.42	2.11 ± 0.47	2.13 ± 0.16	1.77 ± 0.57
Lpl	1.67 ± 0.16^{a}	1.13 ± 0.17^{b}	1.46 ± 0.11^{a}	1.19 ± 0.18^{b}	1.21 ± 0.14^{a}	0.99 ± 0.11^{b}	1.41 ± 0.13^{a}	0.86 ± 0.04^{b}
Pparg$^{\#}$	0.72 ± 0.03	0.95 ± 0.09	1.03 ± 0.20	1.15 ± 0.25	0.70 ± 0.07	1.01 ± 0.11	1.12 ± 0.22	0.71 ± 0.05
Srebf1	2.71 ± 0.19^{a}	1.89 ± 0.10^{b}	2.47 ± 0.33^{a}	1.74 ± 0.15^{b}	2.93 ± 0.53^{a}	2.27 ± 0.17^{b}	2.61 ± 0.40^{a}	1.77 ± 0.20^{b}
Lep$^{\#}$	2.23 ± 0.39^{a}	1.31 ± 0.10^{b}	1.84 ± 0.15^{a}	1.43 ± 0.20^{b}	1.00 ± 0.08	1.03 ± 0.11	1.77 ± 0.13	0.95 ± 0.17

Values are means ± SEM and n=6-9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were analysed using a two-way ANOVA; all comparisons are made within sex group. Different superscripts denote statistical different (P<0.05). A significant interaction of maternal dietary fat content and maternal dietary fatty acid is denoted for †males (P<0.05) and $^{\#}$females (P<0.05). *Fasn*, fatty acid synthase; *Lpl*, lipoprotein lipase; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf1*, sterol regulatory element-binding protein (variant 1c); *Lep*, leptin.

Table 5.4 Offspring lipogenic gene expression in male and female offspring at 8 weeks of age

Experimental Group	Male				Female			
	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**	**High LA (18% Fat)**	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
Liver								
Fasn[†]	1.40 ± 0.14	2.23 ± 0.5	2.20 ± 0.34	1.58 ± 0.13	1.75 ± 0.16	2.45 ± 0.45	1.59 ± 0.29	2.43 ± 0.40
Lpl	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.13 ± 0.01
Pparg	2.57 ± 0.53	2.25 ± 0.30	2.03 ± 0.22	1.71 ± 0.07	1.43 ±0.43	1.16 ± 0.29	0.85 ±0.15	0.94 ± 0.15
Srebf1	1.80 ± 0.21[a]	1.63 ± 0.12[b]	2.12 ± 0.25[a]	1.56 ± 0.07[b]	1.39 ± 0.21	1.27 ± 0.14	1.10 ± 0.18	1.20 ± 0.17
Gonadal Fat								
Fasn	2.24 ± 0.64	2.70 ± 0.53	2.83 ± 0.51	4.03 ± 0.88	1.22 ± 0.35[a]	2.54 ± 0.23[b]	2.06 ± 0.59[a]	4.36 ± 1.10[b]
Lpl	2.91 ± 0.31	3.55 ± 0.52	3.50 ± 0.63	3.12 ± 0.36	2.00 ± 0.38	3.65 ± 0.78	3.06 ± 0.43	3.27 ± 0.53
Pparg	1.02 ± 0.08	0.90 ± 0.11	0.82 ± 0.06	1.00 ± 0.09	0.92 ± 0.07	0.94 ± 0.12	0.89 ± 0.14	1.02 ± 0.11
Srebf1	3.18 ± 0.46	2.98 ± 0.17	3.65 ± 0.47	3.33 ± 0.56	2.48 ± 0.27	2.97 ± 0.33	3.14 ± 0.55	3.81 ± 0.69
Lep	4.79 ± 0.76[a]	4.12 ± 0.52[b]	5.82 ± 0.83[a]	3.58 ± 0.44[b]	2.34 ± 0.53	2.99 ± 0.47	4.44 ± 0.87	3.31 ± 0.58
Retroperitoneal Fat								
Fasn	1.58 ± 0.34	1.85 ± 0.31	1.75 ± 0.17	2.25 ± 0.37	0.64 ± 0.11[a]	2.24 ± 0.43[b]	1.11 ± 0.36[a]	1.53 ± 0.26[b]
Lpl	2.05 ± 0.45	2.01 ± 0.32	2.01 ± 0.19	2.41 ± 0.42	1.30 ± 0.21	2.08 ± 0.28	1.68 ± 0.21	1.54 ± 0.18
Pparg	0.94 ± 0.17	0.92 ± 0.04	0.88 ± 0.05	1.00 ± 0.06	0.92 ± 0.14	1.07 ± 0.09	1.06 ± 0.13	1.01 ± 0.04
Srebf1	1.58 ± 0.30	1.48 ± 0.23	1.64 ± 0.10	1.79 ± 0.24	1.06 ± 0.07	1.81 ± 0.24	1.30 ± 0.12	1.29 ± 0.21
Lep	2.98 ± 0.68	2.62 ± 0.49	2.85 ± 0.33	2.47 ± 0.47	1.49 ± 0.33	1.95 ± 0.21	1.72 ± 0.24	1.49 ± 0.17

Values are means ± SEM and n=6-9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were analysed using a two-way ANOVA; all comparisons are made within sex group. Different superscripts denote statistical different ($P<0.05$). [†] Indicates a significant interaction of maternal dietary fat content and maternal dietary fatty acid in male offspring ($P<0.05$). *Fasn*, fatty acid synthase; *Lpl*, lipoprotein lipase; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf1*, sterol regulatory element-binding protein (variant 1c); *Lep*, leptin.

Figure 5.1 Body weights of (A) male and (B) female offspring post-weaning up to 8 weeks of age exposed to either a high LA (18% fat) diet (closed circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) or a low LA (36% fat) diet (open squares) during gestation and lactation. Offspring weaned onto a chow diet. Values are means ± SEM and n =6–9 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way repeated measures ANOVA and a significant effect of maternal dietary fat content ($P<0.0001$) on body weight was observed for both male and female offspring.

Figure 5.2 Whole blood fatty acid profile in (A) male and (B) female offspring at 4 weeks of age. Values are means ± SEM and n=6-9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA; all comparisons were made within sex group. *Indicates significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$). † indicates a significant interaction effect ($P<0.05$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 5.3 Liver fatty acid profile in (A) male and (B) female offspring at 4 weeks of age. Values are means ± SEM and n=6-9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA; all comparisons were made within sex group. *Indicates significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 5.4 Offspring whole blood fatty acid profile in (A) male and (B) female offspring at 8 weeks of age. Values are means ± SEM and n=6-9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA. All comparisons were made within sex group. *Indicates significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 5.1

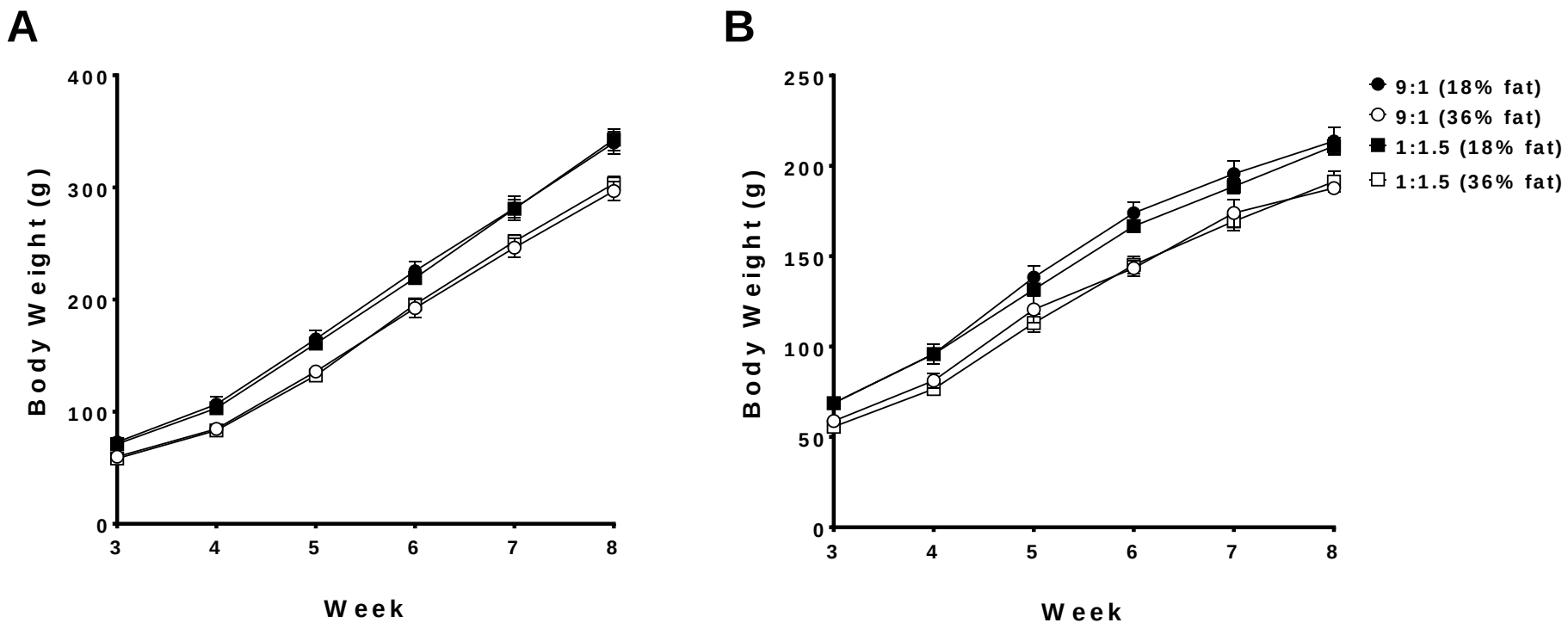


Figure 5.2

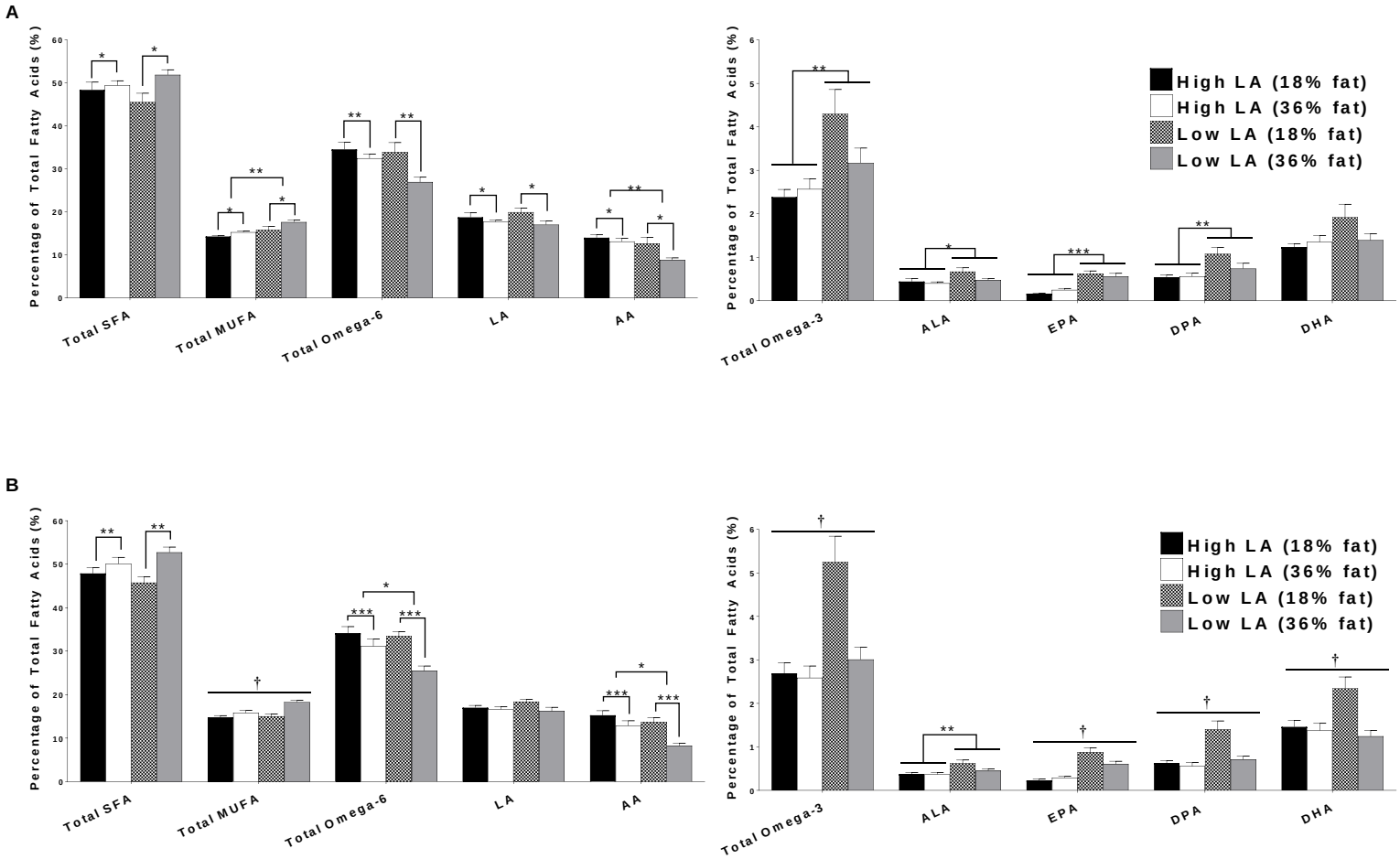
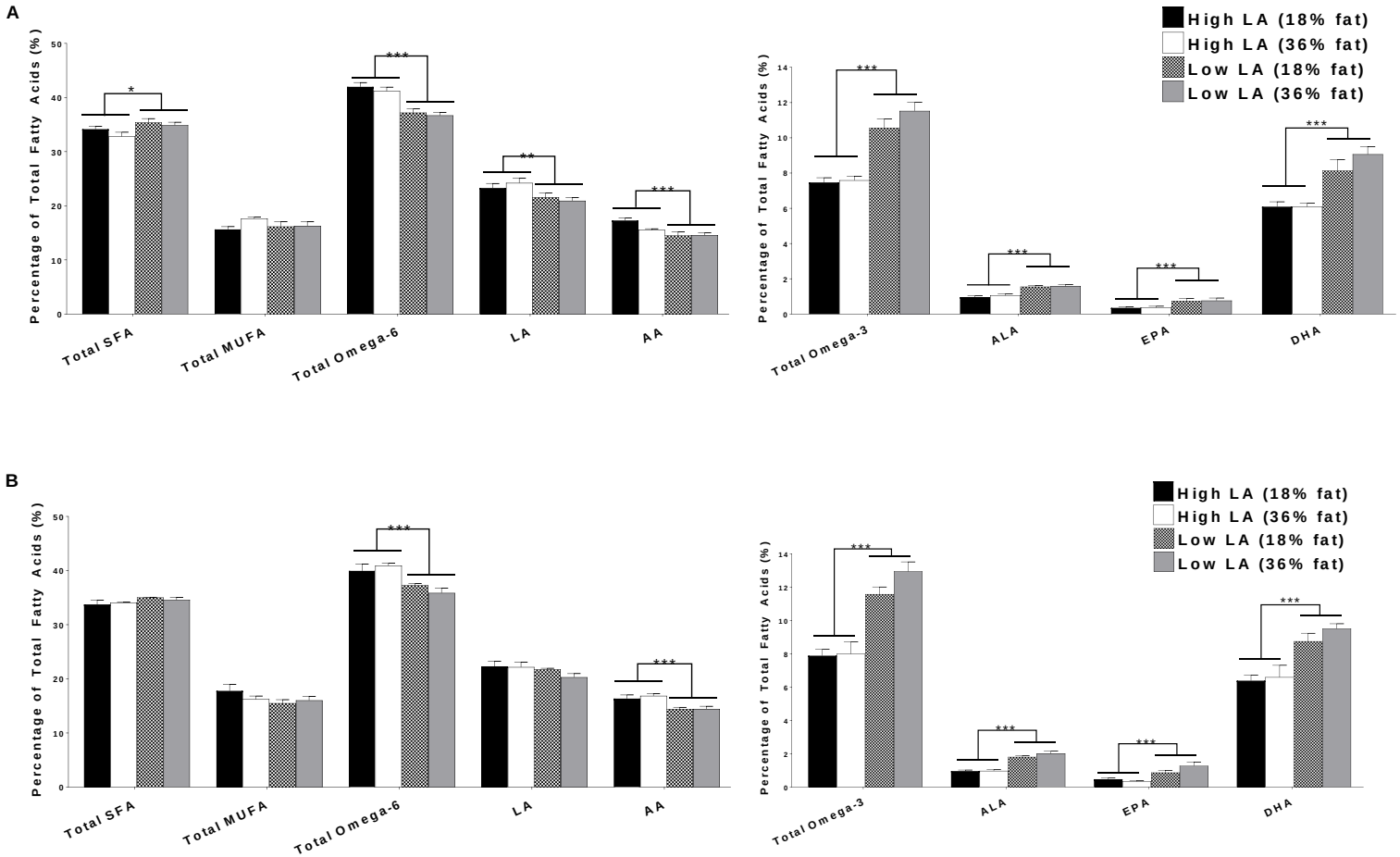

A
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)
B
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)

Figure 5.3


A
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)
B
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
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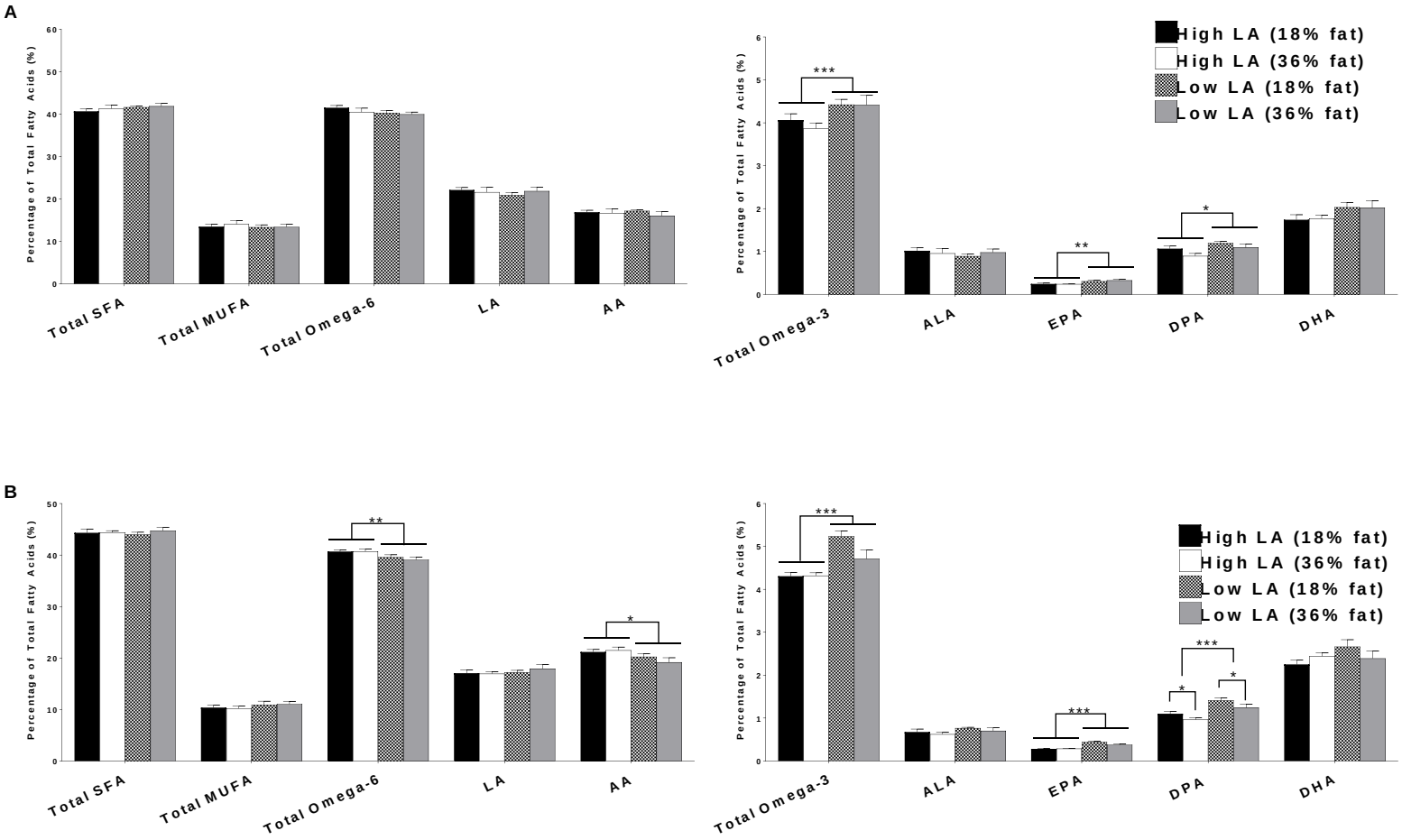
EPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)

Figure 5.4


A
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
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Total Omega-3
ALA
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High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)
B
Percentage of Total Fatty Acids (%)
Total SFA
Total MUFA
Total Omega-6
LA
AA
Total Omega-3
ALA
EPA
DPA
DHA
High LA (18% fat)
High LA (36% fat)
Low LA (18% fat)
Low LA (36% fat)

6 MANUSCRIPT 4: EXPRESSION OF CHOLESTEROL PACKAGING AND TRANSPORT GENES IN HUMAN AND RAT PLACENTA: IMPACT OF OBESITY AND A HIGH FAT DIET

Draycott, SAV, Daniel, Z, Khan, R, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Expression of cholesterol packaging and transport genes in human and rat placenta: impact of obesity and a high-fat diet. *J. Dev. Orig. Health Dis* (accepted manuscript).

Author contributions can be found in Appendix C and accepted manuscript PDF can be found in Appendix F.

6.1 Abstract

Evidence suggests that sub-optimal maternal nutrition has implications for the developing offspring. We have previously shown that exposure to a low-protein diet during gestation was associated with upregulation of genes associated with cholesterol transport and packaging within the placenta. This study aimed to elucidate the effect of altering maternal dietary linoleic acid (LA; omega-6):alpha-linolenic acid (ALA; omega-6) ratios as well as total fat content on placental expression of genes associated with cholesterol transport. The potential for maternal BMI to be associated with expression of these genes in human placental samples was also evaluated. Placentas were collected from 24 Wistar rats at 20d gestation (term=21-22d gestation) that had been fed one of four diets containing varying fatty acid compositions during pregnancy, and from 62 women at the time of delivery. Expression of 14 placental genes associated with cholesterol packaging and transfer were assessed in rodent and human samples by qRT-PCR. In rats, placental mRNA expression of *ApoA2*, *ApoC2*, *Cubn*, *Fgg*, *Mttp* and *Ttr* was significantly elevated (3-30 fold) in animals fed a high LA (36% fat) diet, suggesting increased cholesterol transport across the placenta in this group. In women, maternal BMI was associated with fewer inconsistent alterations in gene expression. In summary, sub-optimal maternal nutrition is associated with alterations in the expression of genes associated with cholesterol transport in a rat model. This may contribute to altered fetal development and potentially programme disease risk in later life. Further investigation of human placenta in response to specific dietary interventions is required.

6.2 Introduction

Maternal nutrition can have a profound impact on fetal development and future physiological function and metabolic health (Langley-Evans, 2015). A number of dietary perturbations, including maternal undernutrition and low protein diets, have been associated with increased risk of obesity and cardiovascular disease in the adult offspring (Le Clair *et al.*, 2009, Langley-Evans, 2013). In the context of the growing epidemic of obesity, focus has shifted towards understanding the effects of nutritional excess and obesity on offspring programming of disease. Studies have consistently demonstrated that these exposures are associated with a substantial increase in the risk of poor metabolic health in the offspring in both humans (Godfrey *et al.*, 2017) and animal models (Ribaroff *et al.*, 2017). There is emerging evidence from animal studies, however, that maternal high-fat diets also have the potential to program metabolic outcomes in the offspring independent of the effects of maternal obesity. In addition, these effects appear to depend not only on the amount of fat in the diet (Ainge *et al.*, 2011), but also on the fatty acid composition (Muhlhausler and Ailhaud, 2013, Ramsden *et al.*, 2013a). The majority of studies to date that have investigated the effects of a maternal high fat diet have utilised diets high in saturated fat. However, due to changes in population level patterns in dietary consumption (Blasbalg *et al.*, 2011, Sioen *et al.*, 2017), attention has now shifted toward the roles of polyunsaturated fats within the diet.

The mechanisms underlying this early life programming of obesity and metabolic disease are not completely understood. However, as the sole interface between the mother and the fetus, structural and functional changes within the placenta have been implicated as playing a key role (Tarrade *et al.*, 2015). Cholesterol is present in every cell of the human body and an adequate supply is therefore critical for supporting normal fetal development. As the precursor for all steroid hormone synthesis, cholesterol also plays an important role in placental function. During pregnancy, the fetus obtains cholesterol via endogenous synthesis as well as transfer across the placenta from the maternal circulation, disturbances to either of these processes have negative impacts on fetal growth, cell proliferation, metabolism and the organisation of tissues (Singh *et al.*, 2013, Fernandez *et al.*, 2005). The endogenous synthesis of cholesterol appears to be most critical for the developing fetus, as defects in this

pathway are known to be lethal (Porter, 2002). Sub-optimal maternal contribution of cholesterol across the placenta, however, has been associated with lower birthweight (Maymunah *et al.*, 2014, Edison *et al.*, 2007) and microcephaly (Edison *et al.*, 2007) in humans, highlighting the importance of this exogenous cholesterol supply.

Transport of cholesterol across the placenta is a complex process in both humans and rodents (Baardman *et al.*, 2013, Woollett, 2011). Briefly, the majority of cholesterol circulates the body in the form of HDL, LDL and VLDL cholesterol, which are associated with specific structural apolipoproteins (*ApoA2*, *ApoB* and *ApoC2* respectively). The layer of trophoblast cells, located closest to maternal circulation, take in LDL and VLDL through their respective receptors. HDL cholesterol can be taken up via a specific receptor (scavenger receptor class B type 1; *SR-B1*) or by binding to proteins such as megalin and cubilin (*Cubn*). Once within the cell, cholesterol is hydrolysed into free cholesterol, bound to sterol carrier proteins and then transferred to the basolateral membrane where it passes through the fetoplacental endothelium. The processes governing cholesterol efflux from the endothelial layer are poorly understood, although it has been shown that exogenous cholesterol is secreted into fetal circulation, through association with various transporters (Stefulj *et al.*, 2009), where it is repackaged into fetal lipoproteins. This process is facilitated by microsomal triglyceride transfer protein (*Mttp*). The finding that the placenta expresses and secretes its own apolipoproteins such as *ApoB* (Madsen *et al.*, 2004) also raises the possibility that cholesterol is repackaged into HDL, LDL and VLDL cholesterol within the placenta itself.

In addition to its critical role in fetal growth and development, there is emerging evidence that alterations in placental cholesterol transfer capacity may also be a contributing factor to metabolic programming. In a previous study (Daniel *et al.*, 2016), we showed providing rats with a low protein diet until day 13 of gestation, a dietary treatment previously associated with programming of obesity, hypertension and glucose intolerance in adult offspring (Langley and Jackson, 1994, Erhuma *et al.*, 2007b), resulted in increased placental expression of a number of genes associated with cholesterol and lipoprotein transport and metabolism in the rat.

Given the similarity in the metabolic phenotype induced by maternal obesity/nutritional excess and low protein diets, we hypothesised that programming of

health and disease is driven by perturbations of a small set of common 'gatekeeper' processes (Swali *et al.*, 2011, McMullen *et al.*, 2012) and changes in placental cholesterol transfer and metabolism may be common mechanisms underlying metabolic programming by different dietary exposures. Therefore, the aim of this current study was to investigate the effect of a high maternal dietary omega-6:omega-3 fatty acid ratio, associated with decreased placental weight (Draycott *et al.*, 2019), against a lower ratio as well as total fat intake, on the expression of genes associated with cholesterol and lipoprotein transport, known to be affected by maternal diet, in the mature placenta of the rat. We also aimed to investigate whether placental expression of these same genes differed according to maternal body mass index (BMI) in a cohort of pregnant women.

6.3 Materials and Methods

6.3.1 Animal experiments and sample collection

This paper reports data from the analysis of placentas that were collected as part of a previous study (Draycott *et al.*, 2019). Virgin female Wistar rats (n = 24; 75-100 g; Charles River, UK) were housed on wood shavings in individually ventilated cages under a 12 h light/12 h dark cycle at a temperature of 20–22 °C and had ad libitum access to food and water throughout the experiment. Female rats were allowed to acclimatise to the unit for 1–2 weeks, during which time they were fed on standard laboratory chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK). After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status. The rats were then randomly assigned to one of 4 dietary groups designed to provide either a high (9:1, high LA) or low (1:1.5 low LA) ratio of linoleic acid (LA) to alpha-linolenic acid (ALA), achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were comparable in all diets, achieved by adjusting the amounts of coconut (SFA source) and macadamia (MUFA source) oils in the diets. For each level of LA, diets containing either 18% or 36% fat by weight were developed. This resulted in four experimental diets; high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat; n =5–7 per dietary group). The list of ingredients and final fatty acid composition of the four experimental diets have been published previously (Draycott *et al.*, 2019).

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Animals were pair housed from the start of the experiment until mating, after confirmation of conception animals were individually housed until completion of the experiment. Animals were maintained on their allocated diet for a four week 'feed-in' period after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Female rats remained on their respective diets until day 20 of gestation (full term = 22 days) at which time rat dams were euthanised by CO_2 asphyxiation and cervical dislocation and fetuses by cervical dislocation and exsanguination. All fetuses were weighed and sexed via measurement of anogenital distance**.** Placentas from male fetuses were collected for analysis and a tail sample from the fetus was collected for sex-genotyping by PCR for the SRY gene (McClive and Sinclair, 2001). Any samples found to be female or inconclusive (n = 5) were not included in placental gene expression analysis. Full details of maternal weight gain, food intake and the effect of the diets on fetal and placental weight are published elsewhere (Draycott *et al.*, 2019).

6.3.2 Human placental sample collection

Ethical approval for the study was obtained from the Derbyshire Research Ethics Committee (Ref: 09/H0401/90). Placental samples were obtained from patients attending the Department of Obstetrics and Gynaecology, Royal Derby Hospital, Derby, UK. Patients provided informed, written consent prior to undergoing elective caesarean section at term gestation (>37 weeks), indications for which were maternal request, previous elective section or breech presentation while cases with diabetes, hypertension, pre-eclampsia were excluded. Placentae, once checked by the midwife and with the cord clamped, were transported to the lab within 20 minutes of delivery, where placental villous samples were taken midway between the cord insertion site and placental periphery and frozen at -80°C prior to extraction of RNA. Participants were stratified based on a BMI measurement taken during an antenatal clinic appointment, resulting in 3 groups of women; BMI <25 kg/m^2 (n=20), BMI 25-35 kg/m^2 (n=21) and BMI >35 kg/m^2 (n=21).

6.3.3 Sample preparation and PCR

RNA was isolated from 20-25mg of crushed snap-frozen human or rat placental tissue using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK) according to manufacturer's instructions. RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Gene targets were chosen based on our previous data (Daniel *et al.*, 2016) where RNASeq analysis of day 13 rat placentas revealed differential expression of 91 genes in response to maternal protein restriction. Ingenuity pathway analysis identified 8 pathways that were significantly affected ($P<0.001$), 6 of which were closely related functionally with a strong emphasis of cholesterol uptake and efflux across the placenta. Genes were selected based upon the ingenuity analysis (*ApoA2, ApoB, ApoC2, Ttr, Fgg, serpin G1* and *Rbp4*). Additional genes were chosen that were shown to be differentially expressed in the protein restricted condition (*Vil1, Gpc3, Prf1, Cubn, Mttp*) but not associated with pathways identified by Ingenuity analysis. *Tagln* and *Tbp* gene targets were also chosen as preliminary RNASeq analysis suggested that they may be sensitive to maternal dietary factors, resulting in 14 target genes for analysis. Real-time PCR primers were designed using Primer Express software (version 1.5; Applied Biosystems) from the RNA sequence, checked using BLAST (National Centre for Biotechnology Information) and were purchased from Sigma (UK). The primer sequences can be found in Supplementary Table 6.4. Real-time PCR was performed on a LightCycler 480 (Roche, Burgess Hill, UK) using the 384 well format. Each reaction contained 5µl of cDNA with the following reagents: 7.5µl SYBR green master mix (Roche), 0.45µl forward and reverse primers (final concentration 0.3µM each) and 1.6µl RNase-free H2O. Samples were pre-incubated at 95°C for 5 min followed by 45 PCR amplification cycles (denaturation, 95°C for 10 seconds; annealing, 60°C for 15 seconds; elongation, 72°C for 15 seconds). Transcript abundance was determined using a standard curve generated from serial dilutions of a pool of cDNA made from all samples. Expression was normalised against the expression of cyclophilin A, which was not significantly different between experimental groups.

6.3.4 Statistical Analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). For animal data, the effect of maternal dietary fatty acid ratio and maternal dietary fat content on placental expression of target genes was assessed using a two-way ANOVA, with dietary fat level and LA:ALA ratio as factors. Human data were analysed using a one-way ANOVA with maternal BMI as a factor. Possible co-variates were identified and corrected for within the analysis. A value of $P<0.05$ was considered statistically significant.

6.4 Results

6.4.1 Rat placenta

The results of the gene expression analyses in the rodent placentas are shown in Table 6.1. Expression was measured for 13 genes (*ApoB* excluded), however, *Tbp* expression was not detectable in any of the samples (data not shown) and so could not be included in the analysis.

Placental *Mttp* mRNA expression was significantly increased (3 fold; $P<0.01$) in dams consuming a high LA compared to a low LA diet, independent of total dietary fat content (Table 6.1). There was a significant interaction between the effects of maternal total dietary fat and LA content in relation to mRNA expression of *ApoA2*, *ApoC2*, *Cubn*, *Fgg*, *Rbp4* and *Ttr* ($P<0.05$), such that expression of these genes was significantly increased in rats consuming the high LA diets, but only when dietary fat content was also high (36% fat w/w). The magnitude of this effect was greatest for *Cubn* (30-fold increase in the high LA (36% fat) group when compared to the low LA (36% fat) group). A similar pattern was observed for other key cholesterol-transport and metabolism genes in the placenta (*Gpc3*, $P=0.072$; *Vil1*, $P=0.054$), with 1.5-7 fold higher expression in placentas of dams exposed to a high LA diet, but only when dietary fat content was also high. Placental expression of *Prf1*, *SerpinG1* and *Tagln* was not affected by either maternal dietary fatty acid ratio or fat content.

6.4.2 Human placenta

The sociodemographic and clinical characteristics of the women who provided placental samples for this study are summarised in Table 6.2. The average age, parity and gestation length of women within this study were similar between women in the three BMI groups, however the birth weights of infants born to women with a BMI >25 kg/m^2 were significantly greater than those of women in the normal BMI range.

The results of the gene expression analyses in the human placentas are shown in Table 6.3. All 14 genes were measured and detected in these samples. Women with a BMI in the 25-35 kg/m^2 range (overweight to obese) exhibited a 2-6 fold higher mRNA expression of *ApoB* in their placental samples compared to women with a BMI either <25 or >35 kg/m^2. The expression of *Rbp4* was significantly lower in placentas obtained from women with a BMI >25 kg/m^2 compared to those with a BMI <25 kg/m^2 (85% down-regulation, P=0.001). A similar pattern was also observed for *Ttr*, with 71-88% down-regulation of expression in women with a BMI >25 kg/m^2, although this was not statistically significant (P=0.053). The mRNA of other placental genes was not different between BMI groups.

6.5 Discussion

This experiment aimed to test the hypothesis that maternal diet, specifically, fatty acid composition and quantity, and obesity would influence the expression of genes associated with cholesterol uptake and transport in rat and human placenta. The results of the rat studies suggested clear effects of maternal dietary fat content and composition, such that maternal consumption of a higher-fat, high LA maternal diet was associated with increased expression of key genes associated with these pathways, suggesting enhanced cholesterol transport to the fetus in this group. In the human study, however, only two of these genes were differentially expressed in placentas from women in different BMI categories, suggesting that maternal obesity had a limited impact on placental cholesterol transport at the level of gene expression.

Within this study, we have shown that exposure to a high LA, high-fat diet resulted in increased expression of genes involved in the formation of apolipoproteins, cholesterol uptake and cholesterol repackaging. These differences were not observed when either the fat content and/or the fatty acid ratio was altered, suggesting a strong interaction

between these variables. Importantly, the effects observed on the placental gene expression profiles show striking resemblances to our previous findings (Daniel *et al.*, 2016) where upregulation of these genes was observed in the placentas of dams exposed to a low protein diet. These data suggest that, not only do these differences persist to a late stage placenta (day 20), but, despite different dietary interventions, the similarities in results suggest a common mechanism of action. The upregulation of key genes in the rat placenta observed in this study suggests a state of increased cholesterol uptake and efflux. While the potential impacts of this in the current study are not clear, it has been demonstrated in previous studies that exposure to excess cholesterol during fetal development can be associated with adverse outcomes. In rodent models, maternal hypercholesterolemia has been associated with growth restriction (Bhasin *et al.*, 2009), altered liver development (El-Sayyad *et al.*, 2014) and atherosclerosis (Napoli *et al.*, 2002, Goharkhay *et al.*, 2007). In humans, maternal hypercholesterolaemia has been associated with the development of fatty streaks in fetal arteries and cholestasis during pregnancy is associated with programming of an overweight, insulin resistant phenotype in the child (Papacleovoulou *et al.*, 2013, Napoli *et al.*, 1997). It will therefore be important in future studies to determine the longer term consequence of the changes in placental gene expression for the postnatal offspring.

Based on the substantial impact of maternal high-fat high LA feeding, a dietary pattern commonly observed in the modern Western diet, we extended our study to determine if there was any evidence to suggest an effect of maternal obesity on cholesterol transfer in the human. There were, however, relatively few differences in the expression of key genes associated with different BMI categories in human placental samples, although there were some subtle differences in the expression of 3 genes (*ApoB, Rbp4* and *Ttr*) between BMI categories. *Ttr* is a protein that binds to and transports *Rbp4*. In the bound state, *Rbp4* is protected from glomerular filtration and so levels of these two proteins are often correlated. As such, the similar patterns of expression across these two genes observed in this study was anticipated. What was surprising, however, was our finding that women with a BMI above the normal range (>25 kg/m^2, overweight or obese) exhibited decreased placental expression of these genes since elevated levels of circulating *Rbp4* have been associated with many of the co-morbidities linked to obesity including hypertension (Solini *et al.*, 2009), insulin

resistance and type 2 diabetes (Yang *et al.*, 2005, Graham *et al.*, 2006). It is important to note, however, that these observations have all been associated with circulating levels of *Rbp4*, whereas we measured gene expression in the placenta. There is limited literature evaluating the role of *Rbp4* within the placenta during pregnancy, particularly in association with maternal obesity. It may be, however, that in obese mothers, placental expression is reduced to compensate for the increased maternal circulating levels and therefore avoiding fetal exposure to high quantities of *Rbp4*. Further experimentation is required to determine expression of *Rbp4* and *Ttr* in both the mother, fetus and the placenta throughout pregnancy and their association with maternal obesity.

Placental *ApoB* expression was increased in women whose BMI was above the normal range (>25 kg/m^2). A study by Dube and colleagues (Dube *et al.*, 2012), showed increased circulating *ApoB* concentrations in new-born infants of obese mothers, compared to mothers of normal weight, in the absence of any difference in maternal circulating concentrations. It is therefore possible that the high *ApoB* concentrations in infants of obese mothers may have been the result of increased placental *ApoB* expression. If this abnormal lipoprotein profile is present in the offspring and persists through childhood, it may contribute to increased risk of cardiovascular disease in later life.

One key limitation of the current study is that direct measurements of cholesterol transport or measurement of the genes of interest at the level of protein were not analysed, and so care must be taken when extrapolating the findings to functional outcomes. Further to this, human participants within this study were stratified based on BMI whereas the animal experiments utilised specific dietary interventions. There are many factors that can affect BMI and, although nutrition is a key element, there is still a huge variety of nutritional habits that can lead to individuals resulting in similar BMIs. As such, assumptions cannot be made about specific nutrient intakes of the women based on these data. Finally, it is important to note that, whilst there are many similarities between placental physiology and function in the rat and humans, there are some key differences. Of particular importance to this study is the difference in circulating progesterone levels at the end of pregnancy. In rodents there is a dramatic decrease in circulating progesterone (Barkley *et al.*, 1979), whereas in humans,

progesterone levels are increased or at least maintained at the time of parturition (Tal *et al.*, 2000). Progesterone is a key steroid produced from cholesterol within the placenta and has been shown to regulate the expression of some genes including *Rbp4* in other tissues (Mullen *et al.*, 2012). As such, careful consideration of the differences in placental hormone production, particularly steroid hormones, between the two species should be made when drawing comparisons.

In conclusion, this study aimed to elucidate whether differences in placental expression of genes involved in cholesterol transport and efflux were associated with altered maternal nutrition in a manner similar to our previous observations in the low-protein model. We demonstrated that exposure to high levels of omega-6 as part of a high-fat diet elicited a similar pattern of placental gene expression, suggesting an increase in cholesterol transport across the placenta. This highlights the potential for a common mechanism by which sub-optimal maternal nutrition during pregnancy alters placental function, and potentially fetal development, resulting in increased risk of disease in later life. We then carried out a preliminary study which aimed to establish if similar alterations were observed in human placentas. Although BMI was associated with some changes in expression, these observations were not consistent and further experimentation is required on placental samples where the specific nutrient intake of the participants are known.

6.6 Tables

Table 6.1 Rat placental gene expression at day 20 gestation

	High LA (18% Fat)	**High LA (36% Fat)**	**Low LA (18% Fat)**	**Low LA (36% Fat)**
*ApoA2**	0.75 ± 0.24	3.24 ± 1.13	0.52 ± 0.08	0.33 ± 0.20
*ApoC2**	0.72 ± 0.27	2.91 ± 1.00	0.56 ± 0.08	0.34 ± 0.21
*Cubn**	0.81 ± 0.35	2.92 ± 1.15	0.35 ± 0.06	0.10 ± 0.04
*Fgg**	0.76 ± 0.30	2.97 ± 1.09	0.36 ± 0.06	0.12 ± 0.05
Gpc3	0.96 ± 0.28	1.46 ± 0.43	0.56 ± 0.09	0.50 ± 0.09
Mttp	0.66 ± 0.25[a]	2.12 ± 0.77[a]	0.28 ± 0.05[b]	0.22 ± 0.09[b]
Prf1	1.65 ± 0.42	0.73 ± 0.14	1.17 ± 0.12	1.15 ± 0.31
*Rbp4**	0.69 ± 0.29	2.34 ± 1.17	0.48 ± 0.12	0.13 ± 0.05
SerpinG1	1.03 ± 0.26	1.00 ± 0.13	0.84 ± 0.21	1.20 ± 0.33
Tagln	0.95 ± 0.13	0.87 ± 0.06	0.67 ± 0.04	1.09 ± 0.17
*Ttr**	0.65 ± 0.22	2.43 ± 0.81	0.52 ± 0.08	0.32 ± 0.17
Vil1	1.23 ± 0.42	2.67 ± 0.97	0.51 ± 0.10	0.54 ± 0.22

* Indicates a significant interaction effect of maternal dietary LA:ALA ratio and total dietary fat content on placental gene expression ($P<0.05$). Different superscript indicate significant differences between groups ($P<0.01$).

Table 6.2 Human participant characteristics

BMI (kg/m^2)	**<25**	**25-35**	**>35**
Age (years)	34.29 ± 1.28	33.55 ± 1.07	31.25 ± 1.04
BMI	21.88 ± 0.36	29.70 ± 0.70	40.10 ± 1.07
Parity	1.05 ± 0.11	0.75 ± 0.14	1.20 ± 0.19
Gestation length (weeks)	38.63 ± 0.23	38.68 ± 0.18	38.65 ± 0.20
Birthweight (g)*	3236 ± 88	3537 ± 112	3565 ± 108
Sex (% male)	45	45	65
N	21	20	20

Data are shown as mean ± SEM for N observations per group. *ANOVA indicated that, with adjustment for gestational age, birthweight was influenced by maternal BMI (P=0.021).

Table 6.3 Human placental gene expression

BMI (kg/m^2)	**BMI <25**	**BMI 25-35**	**BMI >35**
ApoA2	1.04 ± 0.21	1.22 ± 0.27	0.53 ± 0.06
*ApoB***	0.34 ± 0.06	2.19 ± 0.55	1.04 ± 0.25
ApoC2	0.90 ± 0.15	1.02 ± 0.25	0.76 ± 0.13
Cubn	1.22 ± 0.21	1.15 ± 0.15	0.86 ± 0.09
Fgg	0.48 ± 0.14	0.82 ± 0.41	0.65 ± 0.42
Gpc3	0.97 ± 0.20	1.30 ± 0.20	0.83 ± 0.19
Mttp	0.57 ± 0.08	1.23 ± 0.37	0.42 ± 0.08
Prf1	1.14 ± 0.19	0.85 ± 0.10	1.03 ± 0.17
*Rbp4***	0.50 ± 0.13	0.07 ± 0.01	0.08 ± 0.01
SerpinG1	1.45 ± 0.24	0.90 ± 0.07	0.90 ± 0.11
Tagln	0.97 ± 0.11	1.04 ± 0.10	1.11 ± 0.10
Tbp	1.02 ± 0.11	1.16 ± 0.11	1.00 ± 0.07
Ttr	1.92 ± 0.90	0.23 ± 0.08	0.56 ± 0.20
Vil1	1.14 ± 0.20	1.82 ± 0.33	1.01 ± 0.18

* Indicates a significant effect of maternal BMI on placental gene expression (**P<0.01).

Supplementary Table 6.4 Primer sequences used for the determination of gene expression by RT-qPCR

Target Genes		**Rat**	**Human**
Cyclophilin A	FWD:	TGATGGCGAGCCCTTGG	CCCCACCGTGTTCTTCGA
	REV:	TCTGCTGTCTTTGGAACTTTGTC	TGCTGTCTTTGGGACCTTGTC
ApoA2	FWD:	ACTGACTATGGCAAGGATTTGATG	GACCGTGACTGACTATGGCA
	REV:	CTCCTGTGCATTCTGAAAGTAAGC	CAAAGTAAGACTTGGCCTCGG
ApoB	FWD:	-	GGGCAGTGTGATCGCTTCA
	REV:	-	GCGGGTCATGCCTTTGAT
ApoC2	FWD:	GAGCACTTGTTCAGTTACTGGAACTC	GACAGCCGCCCAGAACCT
	REV:	TGCTGTACATGTCCCTCAGTTTCT	TTGCTGTACAAGTCCCTGAGTTTC
Cubn	FWD:	TGCATGTCACCTTCACGTTT	GGCGGATCACCCTAATGTTTAA
	REV:	TGTAAAGCCTCTCCCACTCC	TTGAATACTATCACATGCTCATTGTTG
Fgg	FWD:	CTGGCTGGTGGATGAACAAGT	TCTGGTTGGTGGATGAACAAGT
	REV:	TGGAGTAAGTGCCACCTTGGT	TTTTGAGTAAGTGCCACCTTGGT
Gpc3	FWD:	CGGTTTTCCAAGAGGCCTTT	TCGTGGAGAGATACAGCCAAAA
	REV:	GTAGAGAGACACATCTGTGAAAAATTCA	GGCTCAGGGCCCTTCATT
Mttp	FWD:	TTTTCCTCTGTTTCTTCTCCTCGTA	GAGTGGATCTTCTTCTGCCTACACT
	REV:	AGCTTGTATAGCCGCTCATTATTTAAT	CCAGAACCCGAGTAGAGAATGTCT
Prf1	FWD:	GCTGGCTCCCATTCCAAGAT	CAACTTTGCAGCCCAGAAGAC
	REV:	GCCAGGCGAAAACTGTACATG	TGTGTACCACATGGAAACTGTAGAAG
Rbp4	FWD:	GAGGAAACGATGACCACTGGAT	ACGAGACCGGCCAGATGA
	REV:	TGCAGGCGGCAGGAATA	CACACGTCCCAGTTATTCAAAAGA
SerpinG1	FWD:	GACAGCCTGCCCTCTGACA	GCCCAGACCTGGCCATAAG
	REV:	TTTCTTCCACTTGGCACTCAAG	CCAAGTTGGCGTCACTGTTG
Tagln	FWD:	GGCGTGATTCTGAGCAAGTTG	GGCGTGATTCTGAGCAAGCT
	REV:	CATGGAGGGCGGGTTCTC	GACCATGGAGGGTGGGTTCT
Ttr	FWD:	CCGTTTGCCTCTGGGAAGA	CTTGCTGGACTGGTATTTGTGTCT
	REV:	CCCCTTCCGTGAACTTCTCA	AGAACTTTGACCATCAGAGGACACT
Vil1	FWD:	FWD: AACCAGGCTTTGAACTTCATCAA	CTGAGCGCCCAAGTCAAAG
	REV:	REV: CGGACTCAGCCCCATCATT	CATGGCCTCGATCCTCCATA

Forward (FWD) and reverse (REV) primer sequences designed using Primer Express software (version 1.5; Applied Biosystems)

7 GENERAL DISCUSSION

As discussed in Chapter 1, the well-established phenomenon that changes in the maternal diet can have a lasting impact on the offspring has resulted in the investigation into the role of a wide range of dietary insults (caloric restriction, low protein diets, cafeteria diets, high-fat diets) on offspring disease risk in later life. Animal models have been critical in establishing biological feasibility of the DOHaD hypothesis due to the ability to precisely control dietary intakes in experimental animal studies in a way that is not possible in human trials, and also offer the means to investigate mechanistic drivers. The reproducibility of common offspring phenotypes associated with the MetS, as a result of a wide variety of nutritional insults during early development, provides a strong evidence base to support the role of these dietary influences in early-life programming of metabolic disturbance. It also alludes to the potential of a common underlying mechanism linking different maternal dietary perturbations to increased lifelong risk of obesity and metabolic disease in the offspring (McMullen *et al.*, 2012).

Increases in the intake of LA in Western populations has been documented in several large scale studies (Blasbalg *et al.*, 2011, Ailhaud *et al.*, 2006) and has coincided with large increases in the prevalence of obesity (WHO, 2017a). As such, the number of women of reproductive age who are consuming a high omega-6:omega-3 ratio, in the context of an obesogenic dietary pattern, has increased drastically over the last 60 years and this coincides with increases in the levels of LA in breast milk (Ailhaud *et al.*, 2006). The pro-inflammatory and pro-adipogenic effects of omega-6 fatty acids and their eicosanoid derivatives have been characterized through *in vitro* and experimental animal studies (Massiera *et al.*, 2003, Massiera *et al.*, 2010). This has led to the hypothesis that increased consumption of omega-6 PUFA, relative to omega-3 PUFA, in many Western countries, including the UK, Australia and the US, is contributing to the intergenerational cycle of obesity and prevalence of inflammatory conditions including diabetes as CVD.

Observational studies in humans and experimental animal studies that have attempted to elucidate the role of high omega-6 intakes during pregnancy on the offspring provide some evidence to suggest that this dietary pattern is associated with negative effects on the metabolic health of the progeny (Massiera *et al.*, 2010, Korotkova *et al.*,

2002, Much *et al.*, 2013). However, the number of studies investigating the effect of altering the maternal omega-6:omega-3 ratio on offspring body composition has been limited. Animal studies specifically investigating the impact of increased maternal dietary omega-3 intake on offspring adiposity, however, have produced conflicting results, with some studies reporting decreased (Massiera *et al.*, 2003, Wyrwoll *et al.*, 2006, Donahue *et al.*, 2011) and others reporting increased (Muhlhausler *et al.*, 2011b) offspring fat mass.

The lack of studies in this area and inconsistencies between study designs (including dose and timing of dietary interventions) and reported outcomes has been highlighted as a key issue for drawing clear conclusions regarding the potential role of omega-3 and omega-6 PUFA, and the ratio between them, in cardiometabolic programming (Muhlhausler *et al.*, 2011a). One of the key limitations of most existing animal studies in this area is the use of diets with much higher omega-6:omega-3 PUFA (>200:1; Korotkova *et al.* (2002)) than that encountered in typical human diets. These diets are often also fed in the context of a low overall fat content (~5% energy), owing to the composition of most commercially available rodent diets, making translation of findings to humans difficult. In addition, in most studies, offspring continued to have access to the same diet as their mother after weaning, so that any effects observed cannot be clearly attributed to dietary fatty acid exposure during the gestation and lactation periods (Muhlhausler *et al.*, 2011b, Ibrahim *et al.*, 2009, Muhlhausler *et al.*, 2011a). Finally, no studies prior to this thesis had examined the effects of an increased maternal omega-6:omega-3 ratio on maternal, placental and fetal outcomes before birth. The central aim of this thesis, therefore, was to investigate the role of the omega-6:omega-3 ratio in the maternal diet on offspring health outcomes (such as growth, fat deposition, tissue composition and blood pressure) taking into consideration these limitations and addressing the identified gaps in knowledge.

A key feature of the work described in this thesis was that the diets designed for the experiments were novel and overcame many of the limitations of diets used in previous studies as they were designed to reflect current intakes and recommendations typical of human diets. The LA:ALA ratio of 9:1 was comparable with current estimations of population intakes in the US (Blasbalg *et al.*, 2011) and the ratio of 1:1.5 was selected to represent an ‘ideal’ ratio and was based on maximal conversion of precursor fatty

acids to biologically active long-chain derivatives (Gibson *et al.*, 2011, Lands, 2000, Gibson *et al.*, 2013). The total fat, as well as the composition of SFA, MUFA and total PUFA in the 18% fat w/w diets, was in line with recommended intakes for a healthy diet (FAO and WHO, 2008) and the high-fat diet used in the experiments represented a doubling of this amount, which is more consistent with the higher fat diets in the human context than semi-synthetic high-fat rodent diets.

Taken together the results from maternal whole blood fatty acid analysis in experimental Chapters 3 and 4 confirmed that manipulation of the fatty acid composition of the diet was reflected in the circulating fatty acid profile of the dams. Consistent with previous studies (Tu *et al.*, 2010, Blank *et al.*, 2002, Brenna *et al.*, 2009), we demonstrated that lowering the LA:ALA ratio was not sufficient to produce sustained increases in DHA synthesis at either levels of total PUFA tested. A possible reason for this was that the amount of ALA in the low LA:ALA ratio groups (5-9%en) was sufficient to saturate the Δ6-desaturase enzyme that is utilised for both the conversion of ALA to EPA and DPA to DHA (Figure 1.5). In support of this, the level was notably higher than ALA levels in a number of previous studies that had achieved increases in DHA synthesis (Tu *et al.*, 2010, Gibson *et al.*, 2013, Gibson *et al.*, 2011) where increases in DHA production were only observed when ALA was <2% energy. This adds to the hypothesis that, despite achieving a low LA:ALA ratio in the diet, the conversion of ALA to DHA is inhibited when total ALA (%en) is high (Chan *et al.*, 1993, Mantzioris *et al.*, 1994, Cleland *et al.*, 1992). Despite a maintained increase in the production of EPA in response to a low LA:ALA ratio in these studies, the inability of the diets to increase levels of DHA may help to explain some of the findings as DHA has been shown to be central to many of the beneficial effects observed with respect to omega-3 fatty acids. In future experiments, diets would be designed with the aim to maintain a low LA:ALA ratio, but also to ensure that the overall ALA (% energy) was low.

The finding that diets containing 36% w/w fat did not lead to increases in bodyweight and fat mass in the dams was surprising and may be cause for criticism as to whether these studies are representative of the human population, where consumption of a high-fat diet is often coupled with obesity. However, since maternal obesity has been shown to exert effects independent of the maternal diet (White *et al.*, 2009), the animal model

in this thesis provides an opportunity to examine the effects of dietary manipulation independent of the effects associated with maternal obesity. Therefore, it was possible investigate hypotheses under the assumption that any observed responses were results of the specific changes in dietary composition applied, rather than mediated via the effects on maternal bodyweight and metabolism.

Whilst establishing the maternal state in response to the dietary interventions was important due to the novelty of the formulated diets, assessing the fetal and offspring response was the primary aim of this research. Taken together, the results of Chapters 3 and 6 characterised the fetal and placental response to *in utero* exposure to the experimental diets. To our knowledge this was the first time that the effects of an increased LA:ALA ratio on offspring outcomes before birth had been investigated. When taken in the context of the complete works of this thesis, it appears that the increased fetal weight in response to exposure to a low LA:ALA ratio *in utero* was transient, and that the total fat content of the diet is a much more potent influencer of postnatal growth than the changes in dietary fatty acid ratio investigated in this thesis.

This growth restriction of offspring exposed to a high-fat diet was prevalent at all post-natal life stages and a novel finding of this research. The majority of existing studies reporting decreases in offspring weight in response to a maternal high-fat diet in rodents, utilise diets predominantly rich in SFA which are successful in producing animal models of maternal obesity. What we have shown here is that maternal diets in which SFA is not the predominant fat source, which are a recommendation of UK and Australian governments, still elicit unfavourable outcomes in the offspring that are independent of any maternal weight gain. This is important and could potentially impact on recommendations to expectant mothers around reducing the quantity of fat within their diet even if it has not had any unfavourable phenotypic effects on the individuals themselves.

It is important to note that, before any extrapolations of this research can be made to human populations, comparable studies must first be undertaken in a human cohort. A potential limitation of this study is that reductions in offspring weight in response to a high-fat diet is not a typical feature of human pregnancies. In fact, and as was shown in experimental Chapter 6, exposure to a high-fat diet during gestation and lactation often, but not always (Knudsen *et al.*, 2008), leads to increased offspring weight in

human studies. The observational nature and limitations associated with human studies have resulted in very few, if any, studies that have reliably separated the effects of high-fat diets with maternal obesity. Further to this, accurate information on dietary intakes is difficult to collect and assess. As such, the translation of findings between animal and human studies is difficult.

It is notable that studies focussed on metabolic programming have indicated that the same phenotypes, including hypertension, insulin resistance and/or obesity can be produced by a range of different nutritional insults, raising the possibility of a common underlying mechanism. In addition, these phenotypes can be observed in the adult offspring independent of whether the nutritional treatment results in changes in offspring birthweight. In this thesis, as in most pre-clinical studies in the programming field, the measured endpoints (fat mass, blood pressure etc.) are important indicators of an altered metabolic phenotype but cannot alone indicate whether these changes manifest into a disease state in later life. As such, another limitation of this research was the short duration of the experiment and lack of establishment of an overt phenotype in the offspring. We measured indicators of obesity and hypertension in the offspring, however, in human populations these phenotypes, particularly hypertension, do not often develop until later life. At 8 weeks of age, the oldest offspring in these experiments were equivalent in development to a human teenager. Therefore, in future, these hypotheses should be tested in offspring preferably up to 24 months in age to be able to confidently determine whether a disease state would be established as the animals age.

Whilst maternal dietary fat content was clearly a key determinant of offspring post-natal growth, an altered maternal dietary LA:ALA ratio appeared to have some transient effects on offspring liver weight, hepatic and adipose tissue lipogenesis as well as more long-lasting effects on circulating fatty acid profiles. Many of these observed differences, however, were no longer apparent at 8 weeks of age suggesting that the maternal dietary influence was only capable of exerting a transient effect on all variables measured besides post-natal growth. Whether the prolonged effect of maternal diet on offspring growth, or indeed the transient effects of fat deposition, lipogenesis and liver composition, have repercussions later in life remains to be established. In addition, and despite evidence of the potential effects of increased

omega-3 consumption for weight loss in adult populations, this research established no lasting benefit to decreasing the maternal LA:ALA ratio on offspring adiposity. It was in fact the low LA, low fat diet that resulted in increased fat mass in the offspring. This was consistent with another animal study investigating the effect of maternal DHA supplementation that reported increased fat mass in the offspring (Muhlhausler *et al.*, 2011b). Further to this, recent studies investigating the effects of maternal omega-3 supplementation in human populations, have mostly reported no association between maternal omega-3 intake and early-life adiposity (Li *et al.*, 2018, Vahdaninia *et al.*, 2018). Studies in human populations currently only have the scope to investigate early life indicators of adiposity and later disease risk. As such, whilst there appears to be little advantage to increasing the omega-3 status of pregnant women with respect to offspring risk of obesity and disease at this current time, long-term follow-up of these human cohorts already exposed to alterations in maternal dietary fatty acid composition is key to establishing whether this dietary pattern has any associations with disease in later life. However, the results of this thesis, as well as other studies (Muhlhausler *et al.*, 2011b), suggest this is unlikely to be true.

That many of the observations at 4 weeks of age, such as changes in lipogenic capacity and offspring adiposity, were seemingly 'corrected' for by 8 weeks of age may result in them being regarded as inconsequential. However, other long-term studies investigating maternal dietary insults, such as the well-established low-protein model, have demonstrated that early transient alterations in tissue structure and function, particularly alterations in the expression of lipogenic genes, can be greatly exacerbated in later life and have been shown to be associated with hepatic steatosis in adult rats (Erhuma *et al.*, 2007b). This further reiterates the importance of long-term follow-up studies to test this hypothesis in the context of this research, as well as in the larger perspective of the field. It is important to note that throughout this research many similarities have been drawn to other maternal dietary insults, but particularly to a low protein maternal diet. To assume that the results of this thesis are solely attributable to the total PUFA content and fatty acid composition of the maternal diet may be naïve. As demonstrated by Chapter 3, and particularly 4, alteration in the fat content of the maternal diet led to alterations in the protein intake of dams. Although not measured in this study, this could have resulted in reduced fetal exposure to protein as well as changes in milk composition which could have contributed to the observed differences.

Formulating a diet that only changes a single nutrient component is near impossible and, although useful for examining specific responses, is not representative of normal situations. As such, interpretation of the results of nutritional intervention studies should be open minded as changes in one dietary variable will inevitably affect another.

The extrapolation of rodent data to human cohorts should be carefully considered and the conclusions drawn from the data presented in this thesis may not be true of human populations. However, a significant advantage of utilising an animal model is the ability to investigate the potential mechanisms underlying the observed outcomes. The uniformity of outcomes and considerable conservation of many metabolic processes across mammals suggests that the mechanisms underlying the DOHaD hypothesis would be similar between species. Epigenetic modifications of the fetal genome have been well explored whilst the role of the placenta in fetal programming has been somewhat overlooked. Experimental Chapter 6 highlighted that placental function was sensitive to changes in the maternal diet and adds weight to the concept that dysregulation of nutrient transfer across the placenta may be contributing to fetal programming and long-term health. Human placentas were also examined in an attempt to examine comparability between animal and human studies. Limitations in the retrospective data available from the human participants, however, made comparisons difficult. Despite this, Chapter 6 did highlight that changes in the maternal diet, as inferred from differences in maternal BMI, is associated with altered transcriptome in the human placenta which may lead to altered fetal development and increased disease risk in later life.

In conclusion, previous data supporting the beneficial effects of omega-3 fatty acids on adipocytes have largely been based on *in vitro* studies and have used mature, as opposed to developing, adipocytes (Couet *et al.*, 1997, Hill *et al.*, 2007, Ruzickova *et al.*, 2004). These, as well as studies in animals and humans, often focus on omega-3 dietary supplementation rather than trying to improve the omega-3 status by reducing the amount of omega-6 in the diet (Middleton *et al.*, 2018, Wood *et al.*, 2018, Meyer *et al.*, 2019, Hauner *et al.*, 2009). As such, the findings of this thesis demonstrated for the first time that improving the omega-3 status of offspring, through manipulation of the LA:ALA ratio in the mother, had no lasting beneficial effect on offspring adiposity

in the time-frame of this research. However, whether early-life alterations in tissue function and structure elicit long-term consequences for individuals remains to be established and should be a focus of future studies. Further to this we have identified that a maternal high-fat diet, where saturated fats were not the predominant fat source, leads to restricted offspring growth throughout early life which may impact upon disease risk in later life. Finally, the mechanistic investigations provide a platform for further examination of the placenta and the processes within it that may lead to the identification of potential biomarkers that indicate nutritional programming of disease. A summary of the main results observed in this thesis are summarised in Figure 7.1.

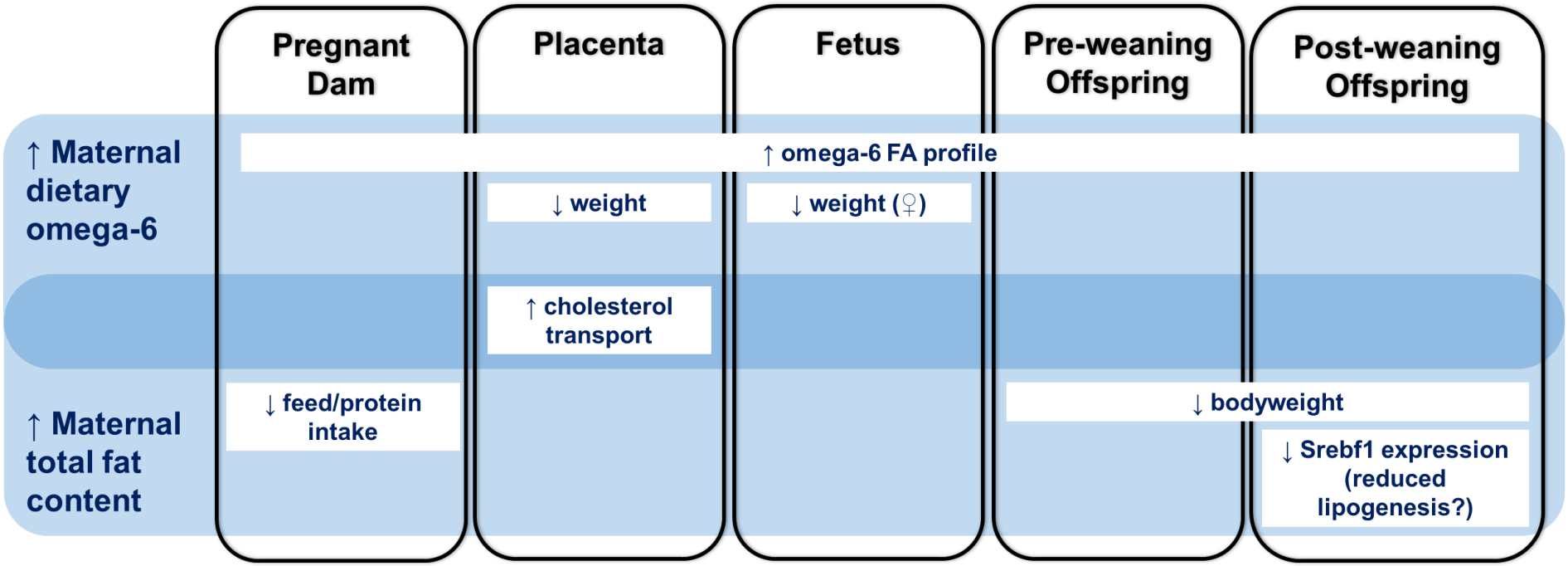

Figure 7.1 Summary of main findings.

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Appendix A

Tecklad Global 18% Protein Rodent Diet (Harlan Laboratories)

2018

Teklad Global 18% Protein Rodent Diet

Product Description- 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2018 does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2018C) and irradiated (2918). For autoclavable diet, refer to 2018S (Sterilizable) or 2018SX (Extruded & Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

*Standard Product Form: **Pellet***

Macronutrients		
Crude Protein	%	18.6
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.5
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.3
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	15.0
Vitamin D_3 [e, g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

Appendix B

Fatty acid composition of Tecklad Global 18% Protein Rodent Diet (Harlan Laboratories)

Fatty Acid	**(% of Total Fatty Acids)**
8:0	-
10:0	-
12:0	-
14:0	0.4
16:0	14.8
18:0	3.8
20:0	0.3
22:0	0.3
24:0	0.2
Total SFA	20.2
18:1n-9	19.0
20:1n-9	0.4
22:1n-9	0.05
16:1n-7	0.4
18:1n-7	1.1
Total MUFA	20.9
18:3n-3 (LA)	6.0
18:2n-6 (ALA)	52.7
Total PUFA	58.7
LA:ALA Ratio	9:1

Appendix C

STATEMENT OF AUTHORSHIP OF PUBLICATIONS

This statement confirms the contribution made by Sally Draycott to jointly authored work

I confirm that Sally Draycott contributed **80%** to the publication:

> **Draycott, SAV**, Liu, G, Daniel, ZC, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Maternal dietary ratio of linoleic acid to alpha-linolenic acid during pregnancy has sex-specific effects on placental and fetal weights in the rat. *Nutrition & Metabolism*, 16, 1.

I confirm that Sally Draycott contributed **80%** to the publication:

> **Draycott, SAV**, George, G, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2020). The effect of maternal dietary fat content and omega-6 to omega-3 ratio on offspring growth and hepatic gene expression in the rat. *British Journal of Nutrition*, 1, 30.

I confirm that Sally Draycott contributed **80%** to the publication:

> **Draycott, SAV,** Daniel, Z, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Omega-6:omega-3 fatty acid ratio and total fat content of the maternal diet alters offspring growth and fat deposition in the rat (prepared manuscript).

I confirm that Sally Draycott contributed **60%** to the publication:

> **Draycott, SAV**, Daniel, Z, Khan, R, Muhlhausler, BS, Elmes, MJ & Langley-Evans, SC (2019). Expression of cholesterol packaging and transport genes in human and rat placenta: impact of obesity and a high-fat diet. *J Dev Orig Health Dis*, 1-6.

Signature:

Date: 24/02/2020

Appendix D

Published article in the journal of Nutrition and Metabolism:

> **Draycott, SAV**, Liu, G, Daniel, ZC, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2019). Maternal dietary ratio of linoleic acid to alpha-linolenic acid during pregnancy has sex-specific effects on placental and fetal weights in the rat. *Nutrition & Metabolism*, 16, 1.

Appendix E

This article has been accepted for publication and will appear in a revised form, subsequent to editorial input by Cambridge University Press, in British Journal of Nutrition published by Cambridge University Press.

Draycott, SAV, George, G, Elmes, MJ, Muhlhausler, BS & Langley-Evans, SC (2020). The effect of maternal dietary fat content and omega-6 to omega-3 ratio on offspring growth and hepatic gene expression in the rat. *British Journal of Nutrition,* 1, 30. https://doi.org/10.1017/S000711452000046X

The effect of maternal dietary fat content and omega-6 to omega-3 ratio on offspring growth and hepatic gene expression in the rat

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Short title: Maternal diet, offspring gene expression.

Key words: Maternal nutrition, omega-6, omega-3, pregnancy, growth restriction

Abstract

Omega-6 fatty acids have been shown to exert pro-adipogenic effects whereas omega-3 fatty acids work in opposition. Increasing intakes of LA (linoleic acid; omega-6) vs ALA (alpha-linolenic acid; omega-3) in Western diets has led to the hypothesis that consumption of this diet during pregnancy may be contributing to adverse offspring health. This study investigated the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) compared to a proposed 'ideal' ratio (~1:1.5), at two total fat levels (18% vs 36% fat w/w), on growth and lipogenic gene expression in the offspring. Female Wistar rats were assigned to one of the four experimental groups throughout gestation and lactation. Offspring were culled at 1 and 2 weeks of age for sample collection. Offspring of dams consuming a -36% fat diet were ~20% lighter than those exposed to a 18% fat diet ($P<0.001$). Male, but not female, liver weight at 1 week was ~13% heavier, and had increased glycogen ($P<0.05$), in offspring exposed to high LA ($P<0.01$). Hepatic expression of lipogenic genes suggested an increase in lipogenesis in male offspring exposed to a 36% fat maternal diet and in female offspring exposed to a low LA diet, via increases in the expression of *Fasn* and *Srebf1*. Sexually dimorphic responses to altered maternal diet appeared to persist until two weeks-of-age. In conclusion, whilst maternal total fat content predominantly affected offspring growth, fatty acid ratio and total fat content had sexually dimorphic effects on offspring liver weight and composition.

Introduction

Accumulating evidence suggests that the nutritional environment experienced by an individual during fetal and early infant development has long-lasting impacts on their metabolic health [1]. In the context of the global epidemic of obesity and nutritional excess, there has been considerable interest in determining the effects of maternal overnutrition on the metabolic health of the offspring. The majority of these studies have utilised animal models and have consistently reported that maternal high-fat feeding during pregnancy has detrimental effects on the metabolic health of both the mother and her offspring [2,3]. As a result, excess maternal fat consumption has been implicated as a key contributor to metabolic programming of long-term health and disease risk.

There is increasing evidence, however, that the impact of a high-fat diet on the metabolic health of the offspring depends not only on the amount of fat in the diet, but also on the fatty acid composition [4,5]. There has been particular interest in the role of two classes of polyunsaturated fatty acids (PUFA), due to the substantive increase in the amounts of omega-6 PUFA, predominately linoleic acid (LA), being consumed in the diets of many Western countries over the past few decades [6,7]. This increase in the intake of LA has not been accompanied by substantial changes in the consumption of omega-3 PUFA such as alpha-linolenic acid (ALA) and has therefore resulted in increases in the ratio of omega-6:omega-3 PUFA consumed in the diets of many Western countries [6,8].

The increasing dominance of omega-6 over omega-3 PUFA in modern Western diets has considerable biological significance, since the omega-6 and omega-3 fatty acid families utilise the same enzymes for production of longer chain bioactive derivatives such as arachidonic acid (AA; omega-6), eicosapentaenoic acid (EPA; omega-3), docosapentaenoic acid (DPA; omega-3) and docosahexaenoic acid (DHA; omega-3), and also compete for incorporation into cell membranes. As a result, excess consumption of LA leads to a decrease in the production and incorporation of omega-3 fatty acids through simple substrate competition, and this effect is exacerbated when total dietary PUFA is high [9,10]. The omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) derivatives also have opposing physiological actions, with those from the omega-3 family predominately exhibiting anti-inflammatory properties (for example via the suppression of the pro-inflammatory transcription factor nuclear factor kappa B and activation of the anti-inflammatory transcription factor peroxisome proliferator activated receptor γ [11]) and those

from the omega-6 family exhibiting more pro-inflammatory and pro-adipogenic properties [12]. This has led to the hypothesis that the increasing ratio of omega-6 to omega-3 fatty acids in modern Western diets may have negative effects on conditions characterised by low-grade inflammation, including obesity and the metabolic syndrome, and may potentially be contributing to an intergenerational cycle of obesity [8].

Data from observational studies in humans and animal models provide supporting evidence that suggests high intakes of omega-6 PUFA during pregnancy could have negative effects on metabolic health of the progeny [13,14,15]. However, the results of these studies have not been consistent. The results of pre-clinical studies are also limited by the use of diets with much higher omega-6:omega-3 PUFA ratios and/or absolute PUFA contents than those encountered in typical human diets. Furthermore, offspring often continue to have access to the same diet as their mother so that any effects observed cannot be clearly attributed to dietary fatty acid exposure during the gestation and lactation periods [16,17,18]. The aim of this study, therefore, was to investigate the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) [6], compared to a proposed 'ideal' ratio of 1:1.5 [19,20] on growth and lipogenic gene expression in the offspring. Since total dietary PUFA intake also influences PUFA metabolism [9,10], we also investigated the effect of feeding each dietary fat ratio at either 18% fat w/w (in line with dietary recommendations [21]) or at a higher fat level of 36% fat w/w. A rat model was utilised to achieve the study objectives by allowing for tight control of dietary manipulation as well as invasive end points.

Materials and Methods

Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Virgin female Wistar rats (n=30; 75-100g; Charles River, UK) were housed on wood shavings in individually ventilated cages under a 12 hour light/12 hour dark cycle at a temperature of 20-22°C and had *ad libitum* access to food and water throughout the experiment. Female rats were allowed to acclimatise to the unit for 1-2 weeks, during which time they were fed standard laboratory chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK). After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status. The rats

were then randomly assigned to one of 4 dietary groups (details provided below). Animals were maintained on their allocated diet for a four week 'pre-feeding' period, after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Animals were housed in individual cages and remained on their respective diets throughout pregnancy and lactation.

Litters were standardised to 8 pups within 24 hours of birth (4 males and 4 females, where possible). At 1 and 2 weeks of age, one randomly selected male and one randomly selected female from each litter were culled via cervical dislocation and exsanguination for blood and tissue collection. At 3 weeks of age, the remaining offspring were weaned and dams were then euthanised by CO_2 asphyxiation and cervical dislocation for collection of maternal blood and tissues. All dams were weighed and had feed intake measured daily throughout the experiment and offspring bodyweight was measured weekly.

Diets

Diets were designed to provide either a high (9:1, high LA) or low (1:1.5, low LA) ratio of LA (cis/cis isomer) to ALA, achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The levels of saturated and monounsaturated fatty acids were comparable in all diets, achieved by adjusting the amounts of coconut (saturated fat source) and macadamia (monounsaturated fat source) oils in the diets. For each level of LA, diets were developed to contain either 18% fat (w/w), in line with government recommendations [21], or 36% fat (w/w) to highlight any additive effects (38.6 vs 63.5% of dietary energy respectively). This resulted in four experimental diets (n=6-9 per dietary group); high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat). The list of ingredients and final fatty acid composition of the four experimental diets have been reported previously [5].

Blood sample and tissue collection

Blood samples were collected from dams prior to the start of the experiment and after the 4 week 'feed-in' period (tail vein sample) and at the end of lactation (via cardiac puncture following CO_2 asphyxiation and cervical dislocation). Truncal blood samples were also collected from one randomly selected male and one randomly selected female at 1 and 2 weeks of age. In all cases, samples of whole blood (~30µl) from non-fasted animals were spotted onto PUFAcoat™ dried blood spot (DBS) collection paper [22], allowed to dry at

room temperature and stored at -20°C for subsequent fatty acid analysis. Maternal tissues were weighed and samples of whole liver, retroperitoneal and gonadal adipose tissues collected. Offspring body and organ weights were measured and whole liver samples were collected from one randomly selected male and female pup at both time points. At 2 weeks of age, samples of gonadal and retroperitoneal fat were also collected from one male and one female pup per litter. All tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until determination of gene expression by quantitative reverse transcriptase PCR (qRT-PCR).

Fatty acid methylation and analysis

Fatty acid composition in maternal and fetal blood was determined as previously described [22]. Briefly, whole DBS samples were directly transesterified with 2ml of 1% H_2SO_4 in methanol and the fatty acid methyl esters (FAME) were extracted with heptane. Samples were separated and analysed by a Hewlett-Packard 6890 gas chromatograph (GC) equipped with a capillary column (30m x 0.25mm) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70; 0.25µm film thickness) which was fitted with a flame ionization detector (FID). FAMEs were identified in unknown samples based on the comparison of retention times with an external lipid standard (Standard 463, Nu-check prep Inc., MN, USA) using Agilent Chemstation software (Agilent Technologies Australia Pty Ltd). Individual fatty acid content was calculated based on peak area and response factors normalised to total fatty acid content and expressed as a percentage of total fatty acids.

Isolation of RNA and cDNA synthesis and quantitative reverse transcription real-time PCR (qRT-PCR)

RNA was isolated from crushed snap-frozen samples of ~25mg of liver using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK). Adipose RNA was extracted, after homogenisation of ~100mg of tissue with MagNA lyser green beads and instrument (Roche Diagnostics Ltd.), using the RNeasy Mini Kit (QIAGEN Ltd., UK). RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Lipogenic pathway and adipokine target genes were chosen based on previous data from our laboratory that indicated that these genes were sensitive to changes in the maternal diet[23] and

included; peroxisome proliferator-activated receptor gamma (*Pparg*), sterol regulatory element-binding protein (variant 1c; *Srebf1*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*) and leptin (*Lep*), with β-actin (*Actb*) as the housekeeper. Primer efficiency ranged from 85%-108% and sequences have previously been published elsewhere [5]. Adipocyte and hepatic gene expression was quantified using SYBR Green (Roche Diagnostics) in a Light-Cycler 480 (Roche Diagnostics). Samples were analysed against a standard curve of a serially diluted cDNA pool to produce quantitative data and expression was normalised to the housekeeping gene using LightCycler® 480 software (version 1.5.1) as previously described [24]. The expression of the housekeeper gene was not different between treatment groups.

Determination of liver DNA, protein and glycogen content

For determination of DNA and protein content of liver samples, approximately 100mg of frozen crushed sample was added to 1ml of 0.05M trisodium citrate buffer. Samples were homogenised and centrifuged at 2500rpm for 10 minutes at 4°C. Supernatant was used for further analyses. DNA concentration (ug/ml) was measured using a Hoechst fluorimetric method and protein content (ug/well), modified for a 96 well plate format, was measured as described by Lowry *et al.* [25]. Measurements were normalised to the exact amount of tissue used for measurements. Liver glycogen was measured using the Colorimetric Glycogen Assay Kit II (Abcam Ltd.) according to manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). The effect of maternal dietary fatty acid ratio and maternal dietary fat content on maternal dependent variables was assessed using a two-way ANOVA, with dietary LA:ALA ratio and dietary fat content as factors and dams were used as the unit of analysis. Where longitudinal data were analysed, as with maternal feed, protein and energy intakes, the impact of maternal dietary LA:ALA ratio and maternal dietary fat content was analysed using a two-way repeated-measures ANOVA. Offspring data were analysed using a two-way ANOVA, with maternal dietary LA:ALA ratio and fat content as factors; where there was no overall effect of sex, male and female offspring data were combined. Where data were not normally distributed, analyses were performed on log10 transformed data. A value of $P<0.05$ was considered to be statistically significant.

Results

Maternal dietary intakes

There were no differences in feed intake of dams between treatment groups before or during pregnancy. During lactation, dams receiving the 36% fat diets had a lower average daily feed intake than those receiving the 18% fat diets, irrespective of dietary LA:ALA ratio ($P<0.001$; Fig 1a). Energy intake was similar between groups throughout the experiment (Fig. 1b). Protein intake prior to and during pregnancy was affected by both dietary LA:ALA ratio and fat content ($P<0.05$; Fig. 1c), however, these effects were small and inconsistent. During lactation, protein intake was affected by dietary fat content only ($P<0.001$; Fig. 1c), such that mothers receiving 36% fat diets consumed 24% less protein on average compared to those consuming 18% fat diets, irrespective of dietary LA:ALA ratio. As expected, all dams consumed more food, energy and protein during lactation than before and during pregnancy regardless of dietary group ($P<0.001$).

Maternal fatty acid profile

There were no differences in the proportions of either saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), omega-6 (Fig. 2a) or omega-3 PUFA (Fig. 2b) in whole blood samples collected from the dams prior to the commencement of dietary intervention. After 4 weeks on their respective diets, the blood fatty acid profiles were significantly different between treatment groups and largely reflected the composition of the experimental diets. Thus, dams fed on high LA diets had higher proportions of LA (1.2 fold) and AA (1.4 fold) compared to those consuming a low LA diet ($P<0.001$; Fig. 2c). Conversely, dams fed the low LA diets had a 5.5 fold higher proportion of ALA and an 8.5 fold higher proportion of EPA compared to those consuming a high LA diet ($P<0.001$; Fig. 2D). These changes were independent of the total fat content of the diet. DPA and DHA levels after the 4 week pre-feeding period were influenced by both dietary LA:ALA ratio and total fat content. Thus, the relative proportions of DPA were higher in dams fed the low LA compared to high LA diets ($P<0.001$), and marginally higher in dams consuming the 18% vs 36% fat diets ($P<0.05$). DHA proportions were also higher in the low LA group ($P<0.001$) but, unlike DPA, were modestly but significantly higher in dams consuming the 36% fat vs 18% fat diets ($P<0.05$; Fig. 2D). Total blood MUFA proportions were higher (1.3-fold) in dams consuming the low LA diet, irrespective of dietary fat content ($P<0.001$; Fig. 2C).

The blood fatty acid profile of the dams at the end of lactation, after a further 6 weeks on their respective experimental diets, were similar to those observed after the first 4 weeks of dietary intervention. A notable difference, however, was that at this time point, relative proportions of DHA, as a percentage of total lipids, were not different between dietary groups (Fig. 2F). LA (1.5-fold), AA (1.8-fold) and total omega-6 (1.5-fold) were all higher in dams consuming a high LA diet irrespective of dietary fat content ($P<0.001$; Fig. 2E). Conversely, total omega-3 levels were 3-fold higher in dams consuming a low LA diet, irrespective of dietary fat content ($P<0.001$). The proportions of ALA were also higher in the groups consuming the low LA diets and in rats consuming the 36% vs 18% fat diets in the low LA group only ($P<0.05$; Fig 2F). DPA proportions were higher in the groups consuming the low LA diets, however, unlike ALA, DPA proportions were lower, rather than higher, in dams consuming the 36% fat diets in the low LA group only ($P<0.001$; Fig. 2F). EPA proportions were higher in groups consuming a low LA diet compared to those consuming a high LA diet ($P<0.001$; Fig. 2F). EPA proportions were also affected by total dietary fat content, and were lower in dams consuming a 36% fat diet compared to an 18% fat diet ($P<0.001$; Fig. 2F). Maternal blood total MUFA levels at the end of lactation were 1.4-fold higher in the dams consuming a low LA diet irrespective of dietary fat content ($P<0.001$; Fig. 2E).

Maternal weight, body composition and gene expression

There were no significant differences in dam bodyweight between dietary groups prior to the commencement of the dietary intervention or at any time during the experiment (data not shown). Dams consuming the 36% fat diets had heavier lungs relative to bodyweight at the end of lactation compared to those consuming the 18% fat diets, independent of the LA:ALA ratio ($P<0.05$). There were no differences in the relative weight of the heart, liver, brain, kidney, gonadal or retroperitoneal fat pads between experimental groups (Table 1).

Analysis of mRNA expression of lipogenic genes indicated that hepatic (3-fold) and gonadal fat (7-fold) expression of *Fasn* was higher in dams consuming an 18% fat diet, compared to those on a 36% fat diet, irrespective of dietary fatty acid ratio ($P<0.01$). The mRNA expression of *Lpl*, *Pparg* and *Srebf1* was not, however, affected by either dietary fat content or ratio in either hepatic or gonadal fat tissues (Table 1). Expression of leptin mRNA in gonadal adipose tissue was not significantly different between treatment groups.

Birth outcomes and offspring bodyweights

There were no differences between dietary groups in terms of litter size or sex ratio of pups (Table 2). Birth weight was lower in offspring of dams fed a 36% fat vs 18% fat diets, independent of the dietary LA:ALA ratio (Table 2). The lower body weight in offspring of dams fed the 36% fat diet persisted during the sucking period such that offspring of dams fed the 36% fat diets remained lighter than offspring of dams fed on 18% fat diets at both 1 and 2 weeks of age; again this was independent of dietary LA:ALA ratio ($P<0.001$; Table 3).

Offspring fatty acid profile

At 1 week of age, proportions of AA (2.1 fold) were lower in the offspring of the low LA compared to high LA dams ($P<0.001$), and in offspring of dams consuming the 36% fat vs 18% fat diets (1.4 fold; $P<0.001$; Fig. 3A). Blood ALA proportions were 5.9 fold higher in offspring of dams in the low LA groups compared to high LA groups ($P<0.001$; Fig. 3B). Offspring EPA and DPA proportions were also higher in the low LA group compared to the high LA group. Blood EPA was also influenced by total dietary fat content, but only in offspring of dams fed the low LA diet, in which EPA levels were lower in offspring of dams fed the 36% fat diets compared to the 18% fat diets (EPA, $P<0.001$; DPA, $P<0.01$; Fig. 3B). DHA proportions were not different between groups at 1 week of age (Fig. 3B). MUFA proportions were higher (1.2-fold) in offspring of dams in the low LA groups ($P<0.001$), consistent with the pattern in maternal blood. However, unlike maternal MUFA, offspring MUFA levels were also affected by maternal dietary fat content and were 1.2-fold higher in offspring of dams fed the 36% fat vs 18% fat diets ($P<0.001$; Fig. 3A). At 1 week of age offspring of dams in the 36% fat diet groups also had lower blood proportions of SFA, irrespective of LA:ALA ratio of the maternal diet ($P<0.01$; Fig. 3A).

The fatty acid profiles of the offspring at 2 weeks of age were similar to those observed at 1 week. Thus, blood AA (1.9 fold) and total omega-6 (1.6 fold) proportions were lower (Fig. 3C) and ALA (6.3 fold), EPA (4.7 fold), DPA (2.4 fold) and total omega-3 PUFA (3-fold) proportions (Fig. 3D) were higher in offspring of dams in the low LA group compared to high LA groups, irrespective of maternal dietary fat content ($P<0.001$). Proportions of LA were higher in offspring of dams fed the 36% fat diets compared to those fed 18% fat diets in the high LA group only ($P<0.05$; Fig 3C), while EPA and DPA proportions were lower in the 36% compared to the 18% fat diet groups, independent of the dietary LA:ALA ratio ($P<0.001$; Fig. 3D). Unlike findings at 1 week of age, the DHA levels in 2 week old offspring

of dams consuming a 36% fat diet were lower (P<0.05) when compared to 18% fat groups, irrespective of maternal dietary fatty acid ratio. As at 1 week, SFA proportions were lower (1.2-fold) in offspring of dams fed a 36% fat diet, independent of the LA:ALA ratio (P<0.001). MUFA proportions were 1.2 fold higher in offspring of dams fed the low LA diets, and 1.2 fold higher in offspring of dams who consumed a 36% fat vs 18% diet (P<0.001; Fig. 3C).

Offspring organ weight and liver composition

At 1 week of age, heart weight relative to bodyweight was higher in female offspring of dams receiving a 36% fat diet compared to the 18% fat diet, independent of the dietary LA:ALA ratio (P<0.05). There were no differences in the relative weight of lung or kidney at 1 week of age and no differences in the relative weight of the heart, lung, liver, gonadal or retroperitoneal fat pads in the offspring at 2 weeks of age between treatment groups in either male or female offspring (Table 3).

Liver weight at 1 week appeared to be influenced by the LA:ALA ratio of the diet to a greater extent than total fat level, at least in males. Thus, male offspring of dams consuming the high LA diets had increased liver weights compared to offspring of dams receiving a low LA diet (P<0.01), irrespective of total dietary fat content. The glycogen content of the livers was also higher in male offspring of dams consuming the high LA diets at 1 week of age (P<0.05). No effect of maternal diet on offspring liver protein or DNA concentration was observed (Table 4). These differences were not present in females at 1 week of age and no differences in glycogen content were observed at two weeks of age in male offspring. DNA concentration in females at two weeks of age was marginally increased (1.1-fold) in offspring exposed to a 36% fat diet, irrespective of maternal dietary fatty acid ratio (P<0.05).

Hepatic gene expression

At 1 week of age, hepatic *Fasn* expression was influenced by maternal dietary intervention in a sex specific manner. Thus, in males, *Fasn* expression was higher in offspring of dams consuming a 36% fat diet irrespective of maternal LA:ALA ratio (P<0.05). In female offspring, however, *Fasn* expression was higher in offspring of dams consuming a low LA diet, independent of dietary fat content (P<0.05). Hepatic *Lpl* mRNA expression in male offspring at 1 week of age was also influenced by maternal dietary fat content, with higher expression in offspring of dams consuming a 36% fat diet vs 18% fat diet (P<0.05). In female

offspring, hepatic *Srebf* expression, similar to that of *Fasn*, was higher in offspring of dams consuming a low LA diet at 1 and 2 weeks of age (P<0.01). Female hepatic expression of *Pparg* was lower in offspring of dams consuming a low LA diet at 2 weeks of age (P<0.05). There were no differences in the expression of *Fasn* or *Lpl* in female offspring, or expression of any hepatic genes in male offspring at this time point (Table 3).

Discussion

This study has demonstrated that altering the fat content and/or LA:ALA ratio of the maternal diet during pregnancy and lactation resulted in significant alteration in the circulating fatty acid profile of dams in the absence of any significant effects on maternal bodyweight or body composition. Exposure to a 36% fat diet during gestation and lactation was, however, associated with lower offspring bodyweight from birth, which persisted to 2 weeks of age. This suggests that increased dietary fat intake during pregnancy and lactation can compromise growth of the progeny, irrespective of the type of fat consumed. In addition, alterations in the fat content and/or composition of the maternal diet had transient effects on offspring body composition and hepatic gene expression, effects which were also sex-specific.

Maternal fatty acid profiles after 4 weeks on the experimental diets largely reflected dietary composition, confirming that the dietary intervention had the desired effect on maternal circulating fatty acid composition. These changes persisted after a further 6 weeks of exposure to the diets and, as expected, the dietary LA:ALA ratio had a greater impact on the maternal blood omega-6 and omega-3 status than total dietary fat content. Consistent with previous studies [5,9,26,27], decreasing the dietary LA:ALA ratio resulted in substantial increases in relative maternal ALA and EPA levels but only a very modest increase in DHA proportions after a 4-week exposure, and no difference compared to the higher LA:ALA ratio after 10 weeks. Interestingly, and independent of dietary LA:ALA ratio, dams appeared to be more efficient at converting DPA to DHA when total dietary fat load was higher. One possibility could be that this is simply a result of the higher amount of substrate (i.e. ALA) available for conversion to the longer chain derivatives such as DPA and DHA in diets containing higher total fat levels. This effect did not, however, persist after a further 6 weeks of dietary exposure, at which point EPA and DPA were lower in dams consuming a low LA 36% fat diet compared to a low LA 18% fat diet. This may be a result of saturation of the PUFA metabolic pathway when total fat, and therefore PUFA, levels were higher [10,28]. This

apparent decrease in capacity to convert ALA through to EPA and DHA during consumption of a 36% fat diet coincides with the decreased protein intake observed in these groups. It is possible that the lower consumption of protein in rats fed on the 36% fat diets may have contributed to reduced conversion of ALA, since previous studies have shown reduced desaturase, particularly Δ6-desaturase, expression in the mammary gland [29] and liver [30] of rats exposed to a low protein diet. Maternal whole blood MUFA proportions appeared to be influenced by dietary LA:ALA ratio, however, this is most likely a result of the slightly higher MUFA content of the low LA diets.

Offspring fatty acid profiles at 1 and 2 weeks of age largely reflected maternal profiles with maternal dietary LA:ALA ratio exhibiting the strongest effect on offspring circulating fatty acid proportions. However, the total fat content of the maternal diet appeared to have a greater influence on the blood fatty acid composition of the offspring as opposed to that observed in the dams. Of particular interest was the finding that the proportion of both EPA and DPA in offspring at 1 week of age were higher in the low LA (18% fat) vs the low LA (36% fat) group, and that this effect persisted at 2 weeks of age despite ALA levels being increased in the low LA (36% fat) group at this time point. DHA was not different between groups at 1 week of age but was lower in offspring exposed to a 36% fat diet at 2 weeks of age. As with the maternal fatty acid profiles, this again may be a result of saturation of the PUFA metabolic pathway at higher total PUFA intakes, and is in line with findings from numerous studies, both human and animal, that indicate that simply increasing the quantity of substrate, i.e. ALA, is not an effective strategy for increasing concentrations of its long-chain derivatives, in particular DHA [26,27,31,32].

The total dietary fat content of the maternal diet also had an influence on the proportion of SFA in the offspring, such that offspring of dams consuming 36% fat diets exhibited lower SFA proportions than offspring of dams consuming the 18% fat diets. Unlike the fetus, where fatty acid composition is largely related to maternal dietary intake, during suckling, offspring fatty acid composition is largely determined by the composition of the milk, which may not fully reflect maternal fatty acid intakes. In a study by Mohammad *et al.* [33], for example, women consuming diets with a higher total fat content (55%en vs. 25%en) exhibited reduced SFA concentrations (C6:0-C14:0) in breast milk but not in maternal plasma. While milk composition was not assessed in the current study, this raises the possibility that SFA content of the milk may have been lower in those dams consuming the 36% fat diets, which could in

turn explain the lower SFA status of the offspring. Alternatively, it may be that increasing the fat content of the diets resulted in an increased conversion of SFA to MUFA, since high-fat feeding has been associated with increased expression of the enzyme responsible for conversion of SFA to MUFA, stearoyl-CoA desaturase 1 (SCD-1) [34] and could therefore be the reason for the observed effect of fat content on offspring MUFA levels in this study. It is important to note, however, that circulating fatty acid profiles are a product of both dietary fatty acid intake as well as tissue fatty acid production and release. Whilst the collection of blood samples from animals in the fed state suggests that the dietary fraction of fatty acids would provide a greater contribution to the fatty acid profile of both dams and offspring, the influence of hepatic synthesis of fatty acids should not be overlooked as a contributor to the observed differences.

Despite significant shifts in maternal fatty acid profiles and increased fat content of the 36% fat diets, we saw no differences in maternal bodyweight or fat deposition. This is consistent with our previous study [5] and is likely a result of the reduced feed intakes of the dams to compensate for the increased energy density of the higher fat diets, a phenomenon consistently seen with dietary intervention trials using rodents [35]. Despite the lack of an effect on maternal weight gain and fat deposition, bodyweight was reduced in offspring of dams receiving a 36% fat diet, irrespective of maternal dietary LA:ALA ratio. This phenotype was consistent across sexes and persisted from birth to 2 weeks of age. Variable results have been reported in this regard with some studies reporting no effects [36,37,38] or increased weight [39]. This finding was, however, consistent with many other studies that reported decreased fetal [40,41], birth [42] and weaning weight [43] in offspring of dams exposed to a 36% fat diet during gestation and lactation periods. The differential effects of different 36% fat diets on offspring growth is likely due to differences in composition of the diet as well as periods of exposure between studies [3]. In those studies that have reported lower offspring weights in offspring fed a high-fat diet, lower protein intakes in dams consuming a high-fat diet have been cited as a likely contributing factor. Further to this, protein restricted diets have been associated with impaired mammary gland development [29,44] leading to impaired milk synthesis [44], and this may also have contributed to reduced offspring growth observed during the suckling period. It is important to note however, that the reduction in protein intake in dams consuming a 36% fat diet in the current study were more modest (10-25%) than those typically used in low-protein diet studies (~50% reduction) [45,46,47,48].

The lower *Fasn* expression in the liver and adipose tissue of dams exposed to a 36% fat diet is consistent with the established role of this enzyme in suppressing lipogenesis in times of energy excess [49]. Surprisingly, this change did not appear to be mediated through changes in maternal *Srebf1* mRNA expression, a known regulator of *Fasn* expression [50]. It is important to note that since only mRNA expression was measured, we cannot comment on any differences in protein expression or activity of this transcription factor although mRNA and protein levels have been shown to be closely correlated [23]. Following this up at the protein level is a major priority for future study. In the offspring, however, hepatic *Fasn* expression was not downregulated by exposure to a maternal 36% fat diet but was actually higher in male offspring of dams consuming the 36% fat compared to the 18% fat diets at 1 week of age and was accompanied by an increase in *Lpl* expression. In female offspring, however, hepatic *Fasn* and *Sbrepf1* expression at 1 week were influenced by maternal dietary fatty acid ratio, rather than total fat content, with both genes upregulated in offspring of dams fed the low LA diets. In both cases, the upregulation of *Sbref1*, *Fasn* and *Lpl* genes would be expected to be associated with an upregulation of both lipogenesis and fatty acid uptake. It is worth mentioning that differences in hepatic expression of lipogenic genes in male offspring were consistently associated with maternal dietary fat content whereas differences in female hepatic expression were consistently associated with maternal dietary fatty acid ratio. This suggests that female offspring are more sensitive to changes in the types of maternal dietary fat whereas male offspring are more sensitive to gross maternal fat consumption. Sex specific effects associated with the programming of disease hypothesis have been frequently reported [51]. The mechanism by which sex influences these effects, however, remains to be elucidated within a larger perspective, as well as within the context of this study.

We found no evidence that these alterations in hepatic gene expression translated to increases in liver weight, however whether there was any effect on hepatic fat content remains to be determined. In both male and female offspring, relative liver weight was increased in offspring of dams fed the high LA diet. In an aim to further elucidate the source of this increased weight, we measured liver DNA, protein and glycogen composition. Similar to liver weight, glycogen levels were increased in offspring of dams fed the high LA diets. This increase in glycogen, however, was not sufficient enough to completely account for the differences observed in liver weight but may be a contributing factor. Consideration of DNA and protein content of the tissue did not indicate significant changes to cell size or number.

More detailed analysis is required to further elucidate the mechanism by which high maternal dietary omega-6 may impact upon offspring liver physiology.

The majority of the hepatic mRNA expression differences, as well as gross differences in liver weight and composition, appeared to be transient and were no longer present at 2 weeks of age. A notable exception was the lower expression of *Srebf1* mRNA and higher expression of *Pparg* in females of dams exposed to a high LA diet compared to the low LA diet, with a similar trend observed in males. Although found in relatively low concentrations in the liver, activation of *Pparg* has been shown to increase hepatic lipid storage and is elevated in models of hepatic steatosis [(52)]. As such, decreased *Pparg* expression can alleviate some of the symptoms of hepatic steatosis leading to a reduced liver weight in conjunction with a reduction in hepatic triglyceride content [(53)]. Thus, our finding that female offspring of dams exposed to a high LA diet tended towards to have an increased liver weight at one week of age followed by increased hepatic *Pparg* expression at two weeks of age may suggest that the increase in *Pparg* expression is a potential response to the increased liver growth observed a week earlier. Alternatively, parallels may be drawn to the effect of low protein diets where fluctuations between an increased and decreased lipogenic capacity, chiefly mediated by altered *Srebf1* expression, occur in early life only to settle into a pattern of upregulated lipogenesis at a later life stage [(23)]. Further studies would be needed to directly evaluate this hypothesis.

In conclusion, we have demonstrated that exposure to a 36% fat diet during gestation and lactation is associated with persistent growth restriction in both male and female offspring irrespective of maternal dietary fatty acid composition. Growth restriction has been associated with a plethora of metabolic disturbances later in life [(54,55,56)] and transient alterations in gene expression have been suggested as a mechanism for programming changes in metabolic processes within tissues as well as the morphology of the tissues themselves [(1)] . In this study, offspring are still exposed to the experimental diets via the dams milk, and further studies in offspring at older ages are required to assess whether the changes in growth, hepatic gene expression and liver weights in the current study are associated with phenotypic changes that persist once offspring are no longer exposed directly to the altered diet composition. In addition, analysis of lipogenic pathway and adipokines targets at the protein level, as well as whole transcriptome analysis, may yield useful information about their regulation and the extent to which these experimental diets programme other metabolic and regulatory pathways in the liver. Finally, the longevity of these perturbations into later life, especially when presented with secondary metabolic challenges such as aging, prolonged high-fat feeding or in the case of female offspring, pregnancy, remains to be elucidated.

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Conflicts of Interest

None

Author Contributions

SCL-E, BSM and MJE participated in study design. SAVD carried out the study (assisted by GG), data analysis and preparation of the manuscript which was revised and approved by SCL-E, BSM, MJE and GG.

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Table 1. Maternal organ weights and gene expression

	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
Bodyweight (g)	308.75 ± 9.82	288.73 ± 14.95	303.29 ± 11.11	302.23 ± 7.85
Heart (% BW)	0.35 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.35 ± 0.01
Lungs (% BW)[a]	0.45 ± 0.02	0.51 ± 0.04	0.42 ± 0.02	0.48 ± 0.02
Kidney (% BW)	0.78 ± 0.02	0.83 ± 0.03	0.82 ± 0.02	0.79 ± 0.02
Liver (% BW)	5.01 ± 0.10	4.80 ± 0.28	5.28 ± 0.10	4.88 ± 0.09
Brain (% BW)	0.59 ± 0.02	0.59 ± 0.02	0.58 ± 0.02	0.59 ± 0.02
Gonadal Fat (% BW)	1.88 ± 0.35	2.02 ± 0.39	1.65 ± 0.19	1.61 ± 0.11
Retroperitoneal Fat (% BW)	0.76 ± 0.13	0.76 ± 0.13	0.76 ± 0.08	0.85 ± 0.15
Liver mRNA Expression				
Fasn[a*]	20.98 ± 6.17	7.03 ± 1.26	25.08 ± 8.12	9.45 ± 1.63
Lpl	0.20 ± 0.04	0.19 ± 0.04	0.19 ± 0.04	0.13 ± 0.01
Pparg	0.63 ± 0.22	0.78 ± 0.18	0.41 ± 0.09	0.70 ± 0.16
Srebf1	3.52 ± 0.91	2.56 ± 0.64	7.85 ± 2.57	3.39 ± 0.61
Gonadal Fat mRNA Expression				
Fasn[a*]	1.29 ± 0.64	0.18 ± 0.05	2.50 ± 1.16	0.37 ± 0.14
Lpl	0.90 ± 0.23	0.87 ± 0.06	1.56 ± 0.41	1.48 ± 0.46
Ppparg	0.91 ± 0.23	1.22 ± 0.20	1.12 ± 0.13	1.16 ± 0.18
Srebf1	1.80 ± 0.48	1.56 ± 0.31	3.43 ± 1.16	2.21 ± 0.62
Lep	0.49 ± 0.08	1.00 ± 0.29	1.10 ± 0.31	1.38 ± 0.25

All values are mean ± SEM and n=6-9 per dietary group. The effect of dietary fatty acid ratio and dietary fat content were assessed using a two-way ANOVA. [a] indicates a significant effect of dietary fat content (P<0.05, *P<0.01). Although not statistically significant there was some evidence that maternal Srebpf1 expression was influenced by the LA (P=0.08) and fat content (P=0.06) of the diet.

Table 2. Birth outcomes

	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
n	6	8	7	9
Litter Size	12.83 ± 1.19	13.00 ± 1.21	13.14 ± 0.40	13.33 ± 1.08
Sex Ratio (male/female)	1.01 ± 0.23	0.97 ± 0.24	1.27 ± 0.28	1.13 ± 0.26
Male Birthweight (g)[a]	6.19 ± 0.53	5.19 ± 0.18	5.66 ± 0.14	5.36 ± 0.11
Female Birthweight (g)[a]	5.60 ± 0.37	4.85 ± 0.21	5.26 ± 0.14	5.07 ± 0.12

All values are mean ± SEM. The effect of dietary fatty acid ratio and dietary fat content was assessed using a two-way ANOVA. [a] indicates a significant effect of maternal dietary fat content ($P<0.05$).

Table 3. Offspring organ weights and hepatic gene expression

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
1 Week Offspring								
Bodyweight (g)	17.52 ± 1.22[a]	12.85 ± 1.16[b]	16.61 ± 0.41[a]	14.20 ± 0.63[b]	15.79 ± 1.11[a]	12.44 ± 1.17[b]	15.66 ± 0.66[a]	13.40 ± 0.56[b]
Heart (% BW)	0.59 ± 0.07	0.67 ± 0.06	0.58 ± 0.04	0.64 ± 0.02	0.56 ± 0.02[a]	0.70 ± 0.06[b]	0.57 ± 0.06[a]	0.69 ± 0.04[b]
Lungs (% BW)	1.87 ± 0.05	1.73 ± 0.04	1.89 ± 0.05	1.90 ± 0.06	1.96 ± 0.11	1.92 ± 0.05	1.88 ± 0.12	1.93 ± 0.05
Kidney (%BW)	1.27 ± 0.08	1.34 ± 0.05	1.19 ± 0.09	1.22 ± 0.02	1.25 ± 0.04	1.38 ± 0.06	1.21 ± 0.10	1.26 ± 0.03
Liver (% BW)	3.17 ± 0.16[a]	3.39 ± 0.13[a]	2.81 ± 0.12[b]	2.89 ± 0.09[b]	3.18 ± 0.10	3.20 ± 0.27	2.96 ± 0.13	2.99 ± 0.05
Liver *Fasn*	0.21 ± 0.08[a]	0.24 ± 0.05[b]	0.18 ± 0.02[a]	0.38 ± 0.04[b]	0.15 ± 0.02[a]	0.22 ± 0.03[a]	0.32 ± 0.06[b]	0.35 ± 0.08[b]
Liver *Lpl*	1.09 ± 0.38[a]	1.26 ± 0.25[b]	0.76 ± 0.15[a]	2.01 ± 0.38[b]	1.26 ± 0.24	1.37 ± 0.46	1.59 ± 0.28	1.81 ± 0.35
Liver *Pparg*	0.40 ± 0.16	0.30 ± 0.07	0.46 ± 0.14	0.38 ± 0.08	0.51 ± 0.11	0.52 ± 0.13	0.62 ± 0.16	0.41 ±0 .06
Liver *Srebpf1*	0.63 ± 0.16	0.56 ± 0.09	0.51 ± 0.10	0.74 ± 0.10	0.44 ± 0.06[a]	0.44 ± 0.05[a]	0.64 ± 0.11[b]	0.80 ± 0.12[b]
2 Week Offspring								
Bodyweight (g)	39.76 ± 1.67[a]	31.78 ± 2.17[b]	39.89 ± 0.59[a]	31.56 ± 1.49[b]	37.77 ± 1.55[a]	31.70 ± 2.05[b]	38.49 ± 0.93[a]	30.75 ± 1.29[b]
Heart (% BW)	0.60 ± 0.01	0.60 ± 0.02	0.61 ± 0.03	0.63 ± 0.01	0.67 ± 0.06	0.67 ± 0.01	0.65 ± 0.03	0.61 ± 0.02
Lungs (% BW)	1.33 ± 0.20	1.26 ± 0.05	1.25 ± 0.07	1.42 ± 0.07	1.28 ± 0.07	1.32 ± 0.05	1.26 ± 0.08	1.32 ± 0.06
Kidney (%BW)	1.05 ± 0.02	1.02 ± 0.03	1.06 ± 0.02	1.00 ± 0.03	1.17 ± 0.04	1.15 ± 0.04	1.14 ± 0.01	1.05 ± 0.02
Gonadal Fat (%BW)	0.22 ± 0.06	0.18 ± 0.02	0.19 ± 0.02	0.18 ± 0.01	0.24 ± 0.02	0.21 ± 0.02	0.23 ± 0.02	0.24 ± 0.03
Retroperitoneal Fat (%BW)	0.36 ± 0.01	0.41 ± 0.04	0.41 ± 0.02	0.39 ± 0.02	0.33 ± 0.03	0.27 ± 0.02	0.29 ± 0.03	0.27 ± 0.01
Liver (% BW)	3.01 ± 0.06	3.08 ± 0.14	3.11 ± 0.02	3.03 ± 0.02	3.18 ± 0.09	3.15 ± 0.09	3.23 ± 0.05	3.01 ± 0.10
Liver *Fasn*	0.17 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.19 ± 0.02	0.20 ± 0.03	0.22 ± 0.03	0.24 ± 0.03
Liver *Lpl*	1.70 ± 0.25[a]	1.81 ± 0.29[b]	1.60 ± 0.13[a]	2.44 ± 0.23[b]	1.25 ± 0.16	1.89 ± 0.16	2.01 ± 0.29	1.81 ± 0.08
Liver *Pparg*	0.56 ± 0.17	0.66 ± 0.10	0.48 ± 0.10	0.42 ± 0.07	0.79 ± 0.25[a]	0.58 ± 0.07[a]	0.31 ± 0.06[b]	0.43 ± 0.07[b]
Liver *Srebf1*	0.74 ± 0.02	0.71 ± 0.08	0.83 ± 0.06	0.80 ± 0.05	0.68 ± 0.07[a]	0.68 ± 0.05[a]	0.83 ± 0.06[b]	0.95 ± 0.10[b]

All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors. Different superscripts denote values which are significantly different ($P<0.05$). n=4-9 per dietary group. All comparisons are made within sex group.

Table 4. Offspring liver composition

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
1 Week Offspring								
Liver DNA (µg/mg tissue)	0.48 ± 0.06	0.54 ± 0.04	0.56 ± 0.06	0.52 ± 0.03	0.51 ± 0.03	0.51 ± 0.04	0.50 ± 0.04	0.52 ± 0.02
Liver Protein (mg/g tissue)	119.2 ± 12.8	137.7 ± 8.9	135.6 ± 5.2	129.8 ± 4.9	123.8 ± 4.5	138.8 ± 8.3	128.6 ± 3.3	129.5 ± 5.2
Liver Glycogen (µg/mg tissue)	12.71 ± 0.70[a]	11.26 ± 1.86[a]	9.72 ± 1.32[b]	8.64 ± 0.76[b]	9.70 ± 0.89	7.73 ± 0.88	9.00 ± 1.43	11.27 ± 1.80
2 Week Offspring								
Liver DNA (µg/mg tissue)	0.59 ± 0.03	0.53 ± 0.05	0.56 ± 0.04	0.51 ± 0.03	0.52 ± 0.02[a]	0.61 ± 0.05[b]	0.52 ± 0.03[a]	0.57 ± 0.01[b]
Liver Protein (mg/g tissue)	115.1 ± 3.6	129.9 ± 13.5	130.2 ± 10.0	117.9 ± 9.4	117.2 ± 9.7	132.3 ± 9.1	120.7 ± 9.4	120.6 ± 6.5
Liver Glycogen (µg/mg tissue)	9.45 ± 0.61	7.48 ± 0.54	8.35 ± 0.98	9.30 ± 1.75	-	-	-	-

All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors. Different superscripts denote values which are significantly different ($P<0.05$). n=4-9 per dietary group. All comparisons are made within sex group.

Figures

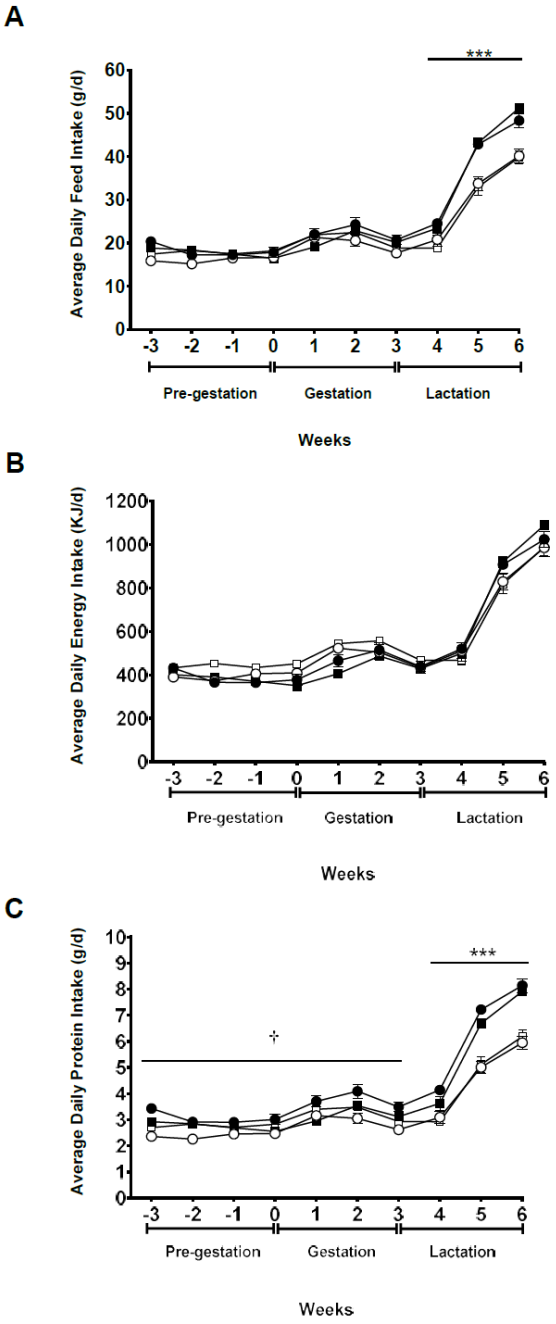

Figure 1. Maternal average daily (A) feed intake, (B) energy intake and (C) protein intake during pre-feeding, pregnancy and lactation fed on either a high LA (18% fat) diet (closed circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) and a low LA (36% fat) diet (open squares). Values are means ± SEM and n=6-9 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way repeated measures ANOVA. * indicates a significant effect of dietary fat content (** P<0.01, *** P<0.001). † indicates a significant interaction between dietary fat content and fatty acid ratio.

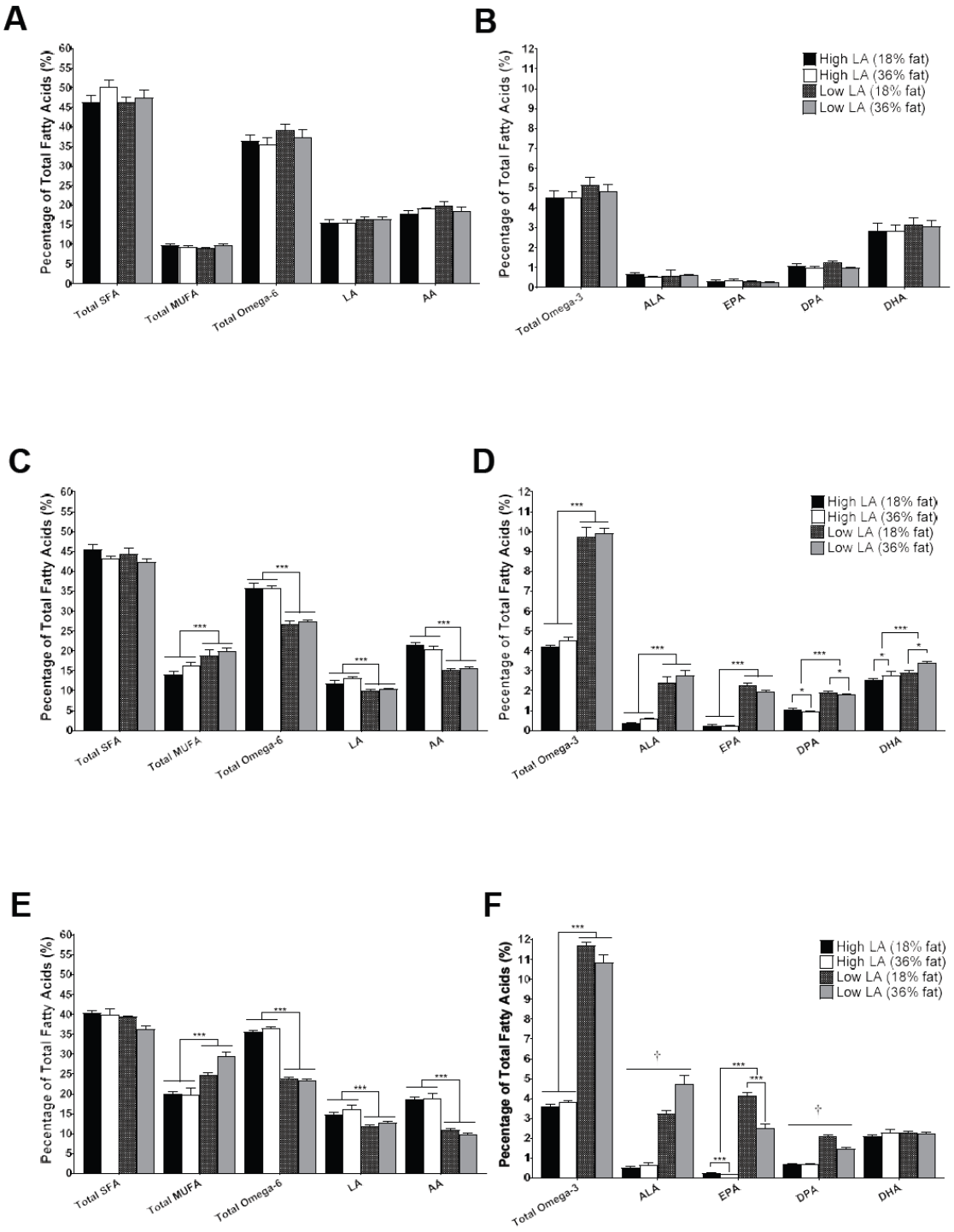

Figure 2. Maternal whole blood fatty acids profile at (A/B) baseline (C/D) after 4 weeks on experimental diet and (D/E) at the end of lactation (3 weeks post-partum). Values are means ± SEM and n=6-9 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way ANOVA (*P<0.05, **P<0.01, ***P<0.001). † indicates a significant interaction effect (P<0.05).

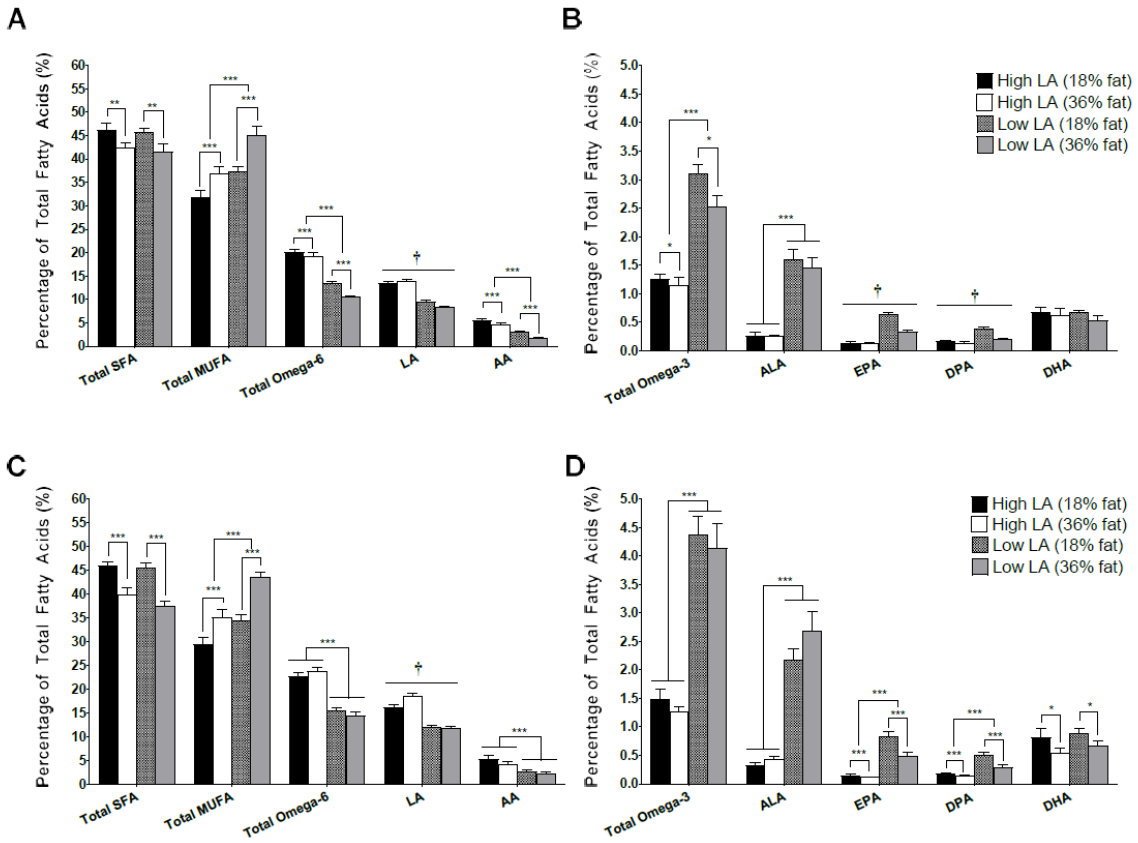

Figure 3. Offspring whole blood fatty acids profile at (A/B) one week of age and (C/D) at two weeks of age. Values are means ± SEM and n=11-17 per group. The effects of maternal dietary fatty acid ratio, maternal dietary fat content and sex were determined using a three-way ANOVA. No effect of sex was found for any of the fatty acids measured and so male and female samples were combined for further analysis. * Indicates significant difference (*P<0.05, **P<0.01, ***P<0.001). † indicates a significant interaction effect (P<0.05).

Appendix F

This paper has been accepted for publication and will appear in a revised form, subsequent to editorial input by Cambridge University Press, in Journal of Developmental Origins of Health and Disease published by Cambridge University press.

Draycott, SAV, Daniel, Z, Khan, R, Muhlhausler, BS, Elmes, MJ & Langley-Evans, SC (2019). Expression of cholesterol packaging and transport genes in human and rat placenta: impact of obesity and a high-fat diet. *J Dev Orig Health Dis*, 1-6. https://doi.org/10.1017/S2040174419000606

Expression of cholesterol packaging and transport genes in human and rat placenta: impact of obesity and a high-fat diet

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Short Title: High-fat diet and placental CHO transport

Abstract

Evidence suggests that sub-optimal maternal nutrition has implications for the developing offspring. We have previously shown that exposure to a low-protein diet during gestation was associated with upregulation of genes associated with cholesterol transport and packaging within the placenta. This study aimed to elucidate the effect of altering maternal dietary linoleic acid (LA; omega-6):alpha-linolenic acid (ALA; omega-6) ratios as well as total fat content on placental expression of genes associated with cholesterol transport. The potential for maternal BMI to be associated with expression of these genes in human placental samples was also evaluated. Placentas were collected from 24 Wistar rats at 20d gestation (term=21-22d gestation) that had been fed one of four diets containing varying fatty acid compositions during pregnancy, and from 62 women at the time of delivery. Expression of 14 placental genes associated with cholesterol packaging and transfer were assessed in rodent and human samples by qRT-PCR. In rats, placental mRNA expression of *ApoA2*, *ApoC2*, *Cubn*, *Fgg*, *Mttp* and *Ttr* was significantly elevated (3-30 fold) in animals fed a high LA (36% fat) diet, suggesting increased cholesterol transport across the placenta in this group. In women, maternal BMI was associated with fewer inconsistent alterations in gene expression. In summary, sub-optimal maternal nutrition is associated with alterations in the expression of genes associated with cholesterol transport in a rat model. This may contribute to altered fetal development and potentially programme disease risk in later life. Further investigation of human placenta in response to specific dietary interventions is required.

Key Words: Cholesterol, maternal nutrition, omega, placenta, pregnancy

Introduction

Maternal nutrition can have a profound impact on fetal development and future physiological function and metabolic health [1]. A number of dietary perturbations, including maternal undernutrition and low protein diets, have been associated with increased risk of obesity and cardiovascular disease in the adult offspring [2]. In the context of the growing epidemic of obesity, focus has shifted towards understanding the effects of nutritional excess and obesity on offspring programming of disease. Studies have consistently demonstrated that these exposures are associated with a substantial increase in the risk of poor metabolic health in the offspring in both humans [3] and animal models [4]. There is emerging evidence from animal studies, however, that maternal high-fat diets also have the potential to program metabolic outcomes in the offspring independent of the effects of maternal obesity. In addition, these effects appear to depend not only on the amount of fat in the diet [5], but also on the fatty acid composition [6]. The majority of studies to date that have investigated the effects of a maternal high fat diet have utilised diets high in saturated fat. However, due to changes in population level patterns in dietary consumption [7], attention has now shifted toward the roles of polyunsaturated fats within the diet.

The mechanisms underlying this early life programming of obesity and metabolic disease are not completely understood. However, as the sole interface between the mother and the fetus, structural and functional changes within the placenta have been implicated as playing a key role [8]. Cholesterol is present in every cell of the human body and an adequate supply is therefore critical for supporting normal fetal development. As the precursor for all steroid hormone synthesis, cholesterol also plays an important role in placental function. During pregnancy, the fetus obtains cholesterol via endogenous synthesis as well as transfer across the placenta from the maternal circulation, disturbances to either of these processes have negative impacts on fetal growth, cell proliferation, metabolism and the organisation of

tissues [9]. The endogenous synthesis of cholesterol appears to be most critical for the developing fetus, as defects in this pathway are known to be lethal [10]. Sub-optimal maternal contribution of cholesterol across the placenta, however, has been associated with lower birthweight [11, 12] and microcephaly [12] in humans, highlighting the importance of this exogenous cholesterol supply.

Transport of cholesterol across the placenta is a complex process in both humans and rodents [13]. Briefly, the majority of cholesterol circulates the body in the form of HDL, LDL and VLDL cholesterol, which are associated with specific structural apolipoproteins (*ApoA2*, *ApoB* and *ApoC2* respectively). The layer of trophoblast cells, located closest to maternal circulation, take in LDL and VLDL through their respective receptors. HDL cholesterol can be taken up via a specific receptor (scavenger receptor class B type 1; *SR-B1*) or by binding to proteins such as megalin and cubilin (*Cubn*). Once within the cell, cholesterol is hydrolysed into free cholesterol, bound to sterol carrier proteins and then transferred to the basolateral membrane where it passes through the fetoplacental endothelium. The processes governing cholesterol efflux from the endothelial layer are poorly understood, although it has been shown that exogenous cholesterol is secreted into fetal circulation, through association with various transporters [14], where it is repackaged into fetal lipoproteins. This process is facilitated by microsomal triglyceride transfer protein (*Mttp*). The finding that the placenta expresses and secretes its own apolipoproteins such as *ApoB* [15] also raises the possibility that cholesterol is repackaged into HDL, LDL and VLDL cholesterol within the placenta itself.

In addition to its critical role in fetal growth and development, there is emerging evidence that alterations in placental cholesterol transfer capacity may also be a contributing factor to metabolic programming. In a previous study[16], we showed providing rats with a low protein

diet until day 13 of gestation, a dietary treatment previously associated with programming of obesity, hypertension and glucose intolerance in adult offspring [17], resulted in increased placental expression of a number of genes associated with cholesterol and lipoprotein transport and metabolism in the rat.

Given the similarity in the metabolic phenotype induced by maternal obesity/nutritional excess and low protein diets, we hypothesised that programming of health and disease is driven by perturbations of a small set of common 'gatekeeper' processes [18] and changes in placental cholesterol transfer and metabolism may be common mechanisms underlying metabolic programming by different dietary exposures. Therefore, the aim of this current study was to investigate the effect of a high maternal dietary omega-6:omega-3 fatty acid ratio, associated with decreased placental weight [19], against a lower ratio as well as total fat intake, on the expression of genes associated with cholesterol and lipoprotein transport, known to be affected by maternal diet, in the mature placenta of the rat. We also aimed to investigate whether placental expression of these same genes differed according to maternal body mass index (BMI) in a cohort of pregnant women.

Materials and Methods

Animal Experiments and sample collection

This paper reports data from the analysis of placentas that were collected as part of a previous study [19]. Virgin female Wistar rats (n = 24; 75-100 g; Charles River, UK) were housed on wood shavings in individually ventilated cages under a 12 h light/12 h dark cycle at a temperature of 20–22 °C and had ad libitum access to food and water throughout the experiment. Female rats were allowed to acclimatise to the unit for 1–2 weeks, during which time they were fed on standard laboratory chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, UK). After acclimatisation, a tail vein blood sample was taken

from each animal for the determination of fatty acid status. The rats were then randomly assigned to one of 4 dietary groups designed to provide either a high (9:1, high LA) or low (1:1.5 low LA) ratio of linoleic acid (LA) to alpha-linolenic acid (ALA), achieved by altering the amounts of flaxseed and sunflower oil included in the fat component of the feed. The levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were comparable in all diets, achieved by adjusting the amounts of coconut (SFA source) and macadamia (MUFA source) oils in the diets. For each level of LA, diets containing either 18% or 36% fat by weight were developed. This resulted in four experimental diets; high LA (18% fat), high LA (36% fat), low LA (18% fat) and low LA (36% fat) (n =5–7 per dietary group). The list of ingredients and final fatty acid composition of the four experimental diets have been published previously [19].

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK. Animals were pair housed from the start of the experiment until mating, after confirmation of conception animals were individually housed until completion of the experiment. Animals were maintained on their allocated diet for a four week 'feed-in' period after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Female rats remained on their respective diets until day 20 of gestation (full term = 22 days) at which time rat dams were euthanised by CO_2 asphyxiation and cervical dislocation and fetuses by cervical dislocation and exsanguination. All fetuses were weighed and sexed via measurement of anogenital distance**.** Placentas from male fetuses were collected for analysis and a tail sample from the fetus was collected for sex-genotyping by PCR for the SRY gene [20]. Any samples found to be female or inconclusive (n = 5) were not included in placental

gene expression analysis. Full details of maternal weight gain, food intake and the effect of the diets on fetal and placental weight are published elsewhere [19].

Human placental sample collection

Ethical approval for the study was obtained from the Derbyshire Research Ethics Committee (Ref: 09/H0401/90). Placental samples were obtained from patients attending the Department of Obstetrics and Gynaecology, Royal Derby Hospital, Derby, UK. Patients provided informed, written consent prior to undergoing elective caesarean section at term gestation (>37 weeks), indications for which were maternal request, previous elective section or breech presentation while cases with diabetes, hypertension, pre-eclampsia, were excluded. Placentae, once checked by the midwife and with the cord clamped, were transported to the lab within 20 minutes of delivery, where placental villous samples were taken midway between the cord insertion site and placental periphery and frozen at -80°C prior to extraction of RNA. Participants were stratified based on a BMI measurement taken during an antenatal clinic appointment, resulting in 3 groups of women; BMI <25 kg/m^2 (n=20), BMI 25-35 kg/m^2 (n=21) and BMI >35 kg/m^2 (n=21).

Sample Preparation and PCR

RNA was isolated from 20-25mg of crushed snap-frozen human or rat placental tissue using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., UK) according to manufacturer's instructions. RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, UK) with random hexamer primers.

Gene targets were chosen based on our previous data [16] where RNASeq analysis of day 13 rat placentas revealed differential expression of 91 genes in response to maternal protein

restriction. Ingenuity pathway analysis identified 8 pathways that were significantly affected ($P<0.001$), 6 of which were closely related functionally with a strong emphasis of cholesterol uptake and efflux across the placenta. Genes were selected based upon the ingenuity analysis (*ApoA2, ApoB, ApoC2, Ttr, Fgg, serpin G1* and *Rbp4*). Additional genes were chosen that were shown to be differentially expressed in the protein restricted condition (*Vil1, Gpc3, Prf1, Cubn, Mttp*) but not associated with pathways identified by Ingenuity analysis. *Tagln* and *Tbp* gene targets were also chosen as preliminary RNASeq analysis suggested that they may be sensitive to maternal dietary factors, resulting in 14 target genes for analysis. Real-time PCR primers were designed using Primer Express software (version 1.5; Applied Biosystems) from the RNA sequence, checked using BLAST (National Centre for Biotechnology Information) and were purchased from Sigma (UK). The primer sequences can be found in supplementary table S1. Real-time PCR was performed on a Lightcycler 480 (Roche, Burgess Hill, UK) using the 384 well format. Each reaction contained 5µl of cDNA with the following reagents: 7.5µl SYBR green master mix (Roche), 0.45µl forward and reverse primers (final concentration 0.3µM each) and 1.6µl RNase-free H2O. Samples were pre-incubated at 95°C for 5 min followed by 45 PCR amplification cycles (denaturation, 95°C for 10 seconds; annealing, 60°C for 15 seconds; elongation, 72°C for 15 seconds). Transcript abundance was determined using a standard curve generated from serial dilutions of a pool of cDNA made from all samples. Expression was normalised against the expression of cyclophilin A, which was not significantly different between experimental groups.

Statistical Analysis

Data are presented as mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc.). For animal data the effect of maternal dietary fatty acid ratio and maternal dietary fat content on placental expression of target genes was assessed using a two-way ANOVA, with dietary fat level and LA:ALA ratio as factors.

Human data were analysed using a one-way ANOVA with maternal BMI as a factor. Possible co-variates were identified and corrected for within the analysis. A value of $P<0.05$ was considered statistically significant.

Results

Rat placenta

The results of the gene expression analyses in the rodent placentas is shown in Table 1. Expression was measured for 13 genes (*ApoB* excluded), however, *Tbp* expression was not detectable in any of the samples (data not shown) and so could not be included in the analysis.

Placental *Mttp* mRNA expression was significantly increased (3 fold; $P<0.01$) in dams consuming a high LA compared to a low LA diet, independent of total dietary fat content (Table 1). There was a significant interaction between the effects of maternal total dietary fat and LA content in relation to mRNA expression of *ApoA2*, *ApoC2*, *Cubn*, *Fgg*, *Rbp4* and *Ttr* ($P<0.05$), such that expression of these genes was significantly increased in rats consuming the high LA diets, but only when dietary fat content was also high (36% fat w/w). The magnitude of this effect was greatest for *Cubn* (30-fold increase in the high LA (36% fat) group when compared to the low LA (36% fat) group). A similar pattern was observed for other key cholesterol-transport and metabolism genes in the placenta (*Gpc3*, $P=0.072$; *Vil1*, $P=0.054$), with 1.5-7 fold higher expression in placentas of dams exposed to a high LA diet, but only when dietary fat content was also high. Placental expression of *Prf1*, *SerpinG1* and *Tagln* was not affected by wither maternal dietary fatty acid ratio or fat content.

Human placenta

The sociodemographic and clinical characteristics of the women who provided placental samples for this study are summarised in Table 2. The average age, parity and gestation

length of women within this study was similar between women in the three BMI groups, however the birth weights of infants born to women with a BMI >25 kg/m^2 were significantly greater than those of women in the normal BMI range.

The results of the gene expression analyses in the human placentas is shown in Table 3. All 14 genes were measured and detected in these samples. Women with a BMI in the 25-35 kg/m^2 range (overweight to obese) exhibited a 2-6 fold higher mRNA expression of *ApoB* in their placental samples compared to women with a BMI either <25 or >35 kg/m^2. The expression of *Rbp4* was significantly lower in placentas obtained from women with a BMI >25 kg/m^2 compared to those with a BMI <25 kg/m^2 (85% down-regulation, P=0.001). A similar pattern was also observed for *Ttr*, with 71-88% down-regulation of expression in women with a BMI >25 kg/m^2, although this did was not statistically significant (P=0.053). The mRNA of other placental genes was not different between BMI groups.

Discussion

This experiment aimed to test the hypothesis that maternal diet, specifically, fatty acid composition and quantity, and obesity would influence the expression of genes associated with cholesterol uptake and transport in rat and human placenta. The results of the rat studies suggested clear effects of maternal dietary fat content and composition, such that maternal consumption of a higher-fat, high LA maternal diet was associated with increased expression of key genes associated with these pathways, suggesting enhanced cholesterol transport to the fetus in this group. In the human study, however, only two of these genes were differentially expressed in placentas from women in different BMI categories, suggesting that maternal obesity had a limited impact on placental cholesterol transport at the level of gene expression.

Within this study we have shown that exposure to a high LA, high-fat diet resulted in increased expression of genes involved in the formation of apolipoproteins, cholesterol

uptake and cholesterol repackaging. These differences were not observed when either the fat content and/or the fatty acid ratio was altered, suggesting a strong interaction between these variables. Importantly, the effects observed on the placental gene expression profiles show striking resemblances to our previous findings [16] where upregulation of these genes was observed in the placentas of dams exposed to a low protein diet. These data suggest that, not only do these differences persist to a late stage placenta (day 20), but, despite different dietary interventions, the similarities in results suggest a common mechanism of action. The upregulation of key genes in the rat placenta observed in this study suggests a state of increased cholesterol uptake and efflux. While the potential impacts of this in the current study are not clear, it has been demonstrated in previous studies that, exposure to excess cholesterol during fetal development can be associated with adverse outcomes. In rodent models, maternal hypercholesterolemia has been associated with growth restriction [21], altered liver development [22] and atherosclerosis [23]. In humans, maternal hypercholesterolaemia has been associated with the development of fatty streaks in fetal arteries and cholestasis during pregnancy is associated with programming of an overweight, insulin resistant phenotype in the child [24]. It will therefore be important in future studies to determine the longer term consequence of the changes in placental gene expression for the postnatal offspring.

Based on the substantial impact of maternal high-fat high LA feeding, a dietary pattern commonly observed in the modern Western diet, we extended our study to determine if there was any evidence to suggest an effect of maternal obesity on cholesterol transfer in the human. There were, however, relatively few differences in the expression of key genes associated with different BMI categories in human placental samples, although there were some subtle differences in the expression of 3 genes (*ApoB*, *Rbp4* and *Ttr*) between BMI categories. *Ttr* is a protein that binds to and transports *Rbp4*. In the bound state, *Rbp4* is

protected from glomerular filtration and so levels of these two proteins are often correlated. As such, the similar patterns of expression across these two genes observed in this study was anticipated. What was surprising, however, was our finding that women with a BMI above the normal range (>25 kg/m^2, overweight or obese) exhibited decreased placental expression of these genes since elevated levels of circulating *Rbp4* have been associated with many of the co-morbidities linked to obesity including hypertension [25], insulin resistance and type 2 diabetes [26]. It is important to note, however, that these observations have all been associated with circulating levels of *Rbp4*, whereas we measured gene expression in the placenta. There is limited literature evaluating the role of *Rbp4* within the placenta during pregnancy, particularly in association with maternal obesity. It may be, however, that in obese mothers, placental expression is reduced to compensate for the increased maternal circulating levels and therefore avoiding fetal exposure to high quantities of *Rbp4*. Further experimentation, is required to determine expression of *Rbp4* and *Ttr* in both the mother, fetus and the placenta throughout pregnancy and their association with maternal obesity.

Placental *ApoB* expression was increased in women whose BMI was above the normal range (>25 kg/m^2). A study by Dube and colleagues [27], showed increased circulating *ApoB* concentrations in new-born infants of obese mothers, compared to mothers of normal weight, in the absence of any difference in maternal circulating concentrations. It is therefore possible that the high *ApoB* concentrations in infants of obese mothers may have been the result of increased placental *ApoB* expression. If this abnormal lipoprotein profile is present in the offspring and persists through childhood it may contribute to increased risk of cardiovascular disease in later life.

One key limitation of the current study is that direct measurements of cholesterol transport or measurement of the genes of interest at the level of protein were not analysed, and so care must be taken when extrapolating the findings to functional outcomes. Further to this, human

participants within this study were stratified based on BMI whereas the animal experiments utilised specific dietary interventions. There are many factors that can affect BMI and, although nutrition is a key element, there is still a huge variety of nutritional habits that can lead to individuals resulting in similar BMIs. As such, assumptions cannot be made about specific nutrient intakes of the women based on this data. Finally, it is important to note that, whilst there are many similarities between placental physiology and function in the rat and humans, there are some key differences. Of particular importance to this study is the difference in circulating progesterone levels at the end of pregnancy. In rodents there is a dramatic decrease in circulating progesterone [28], whereas in humans, progesterone levels are increased or at least maintained at the time of parturition [29]. Progesterone is a key steroid produced from cholesterol within the placenta and has been shown to regulate the expression of some genes including *Rbp4* in other tissues [30]. As such, careful consideration of the differences in placental hormone production, particularly steroid hormones, between the two species should be made when drawing comparisons.

In conclusion, this study aimed to elucidate whether differences in placental expression of genes involved in cholesterol transport and efflux were associated with altered maternal nutrition in a manner similar to our previous observations in the low-protein model. We demonstrated that exposure to high levels of omega-6 as part of a high-fat diet elicited a similar pattern of placental gene expression, suggesting an increase in cholesterol transport across the placenta. This highlights the potential for a common mechanism by which sub-optimal maternal nutrition during pregnancy alters placental function, and potentially fetal development, resulting in increased risk of disease in later life. We then carried out a preliminary study which aimed to establish if similar alterations were observed in human placentas. Although BMI was associated with some changes in expression, these observations

306 were not consistent and further experimentation is required on placental samples where the
307 specific nutrient intake of the participants are known.

308

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Conflicts of interest

None

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the Derbyshire Research Ethics Committee (Ref. 09/H0401/90).

The authors assert that all animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK**.**

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444 **Table 1** Rat placental gene expression at day 20 gestation

	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
ApoA2*	0.75 ± 0.24	3.24 ± 1.13	0.52 ± 0.08	0.33 ± 0.20
ApoC2*	0.72 ± 0.27	2.91 ± 1.00	0.56 ± 0.08	0.34 ± 0.21
Cubn*	0.81 ± 0.35	2.92 ± 1.15	0.35 ± 0.06	0.10 ± 0.04
Fgg*	0.76 ± 0.30	2.97 ± 1.09	0.36 ± 0.06	0.12 ± 0.05
Gpc3	0.96 ± 0.28	1.46 ± 0.43	0.56 ± 0.09	0.50 ± 0.09
Mttp	0.66 ± 0.25^{a}	2.12 ± 0.77^{a}	0.28 ± 0.05^{b}	0.22 ± 0.09^{b}
Prf1	1.65 ± 0.42	0.73 ± 0.14	1.17 ± 0.12	1.15 ± 0.31
Rbp4*	0.69 ± 0.29	2.34 ± 1.17	0.48 ± 0.12	0.13 ± 0.05
SerpinG1	1.03 ± 0.26	1.00 ± 0.13	0.84 ± 0.21	1.20 ± 0.33
Tagln	0.95 ± 0.13	0.87 ± 0.06	0.67 ± 0.04	1.09 ± 0.17
Ttr*	0.65 ± 0.22	2.43 ± 0.81	0.52 ± 0.08	0.32 ± 0.17
Vil1	1.23 ± 0.42	2.67 ± 0.97	0.51 ± 0.10	0.54 ± 0.22

445 * Indicates a significant interaction effect of maternal dietary LA:ALA ratio and total dietary

446 fat content on placental gene expression ($P<0.05$).

447 Different superscript indicate significant differences between groups ($P<0.01$).

448 **Table 2** Human participant characteristics

BMI (kg/m^2)	<25	25-35	>35
Age (years)	34.29 ± 1.28	33.55 ± 1.07	31.25 ± 1.04
BMI	21.88 ± 0.36	29.70 ± 0.70	40.10 ± 1.07
Parity	1.05 ± 0.11	0.75 ± 0.14	1.20 ± 0.19
Gestation length (weeks)	38.63 ± 0.23	38.68 ± 0.18	38.65 ± 0.20
Birthweight (g)*	3236 ± 88	3537 ± 112	3565 ± 108
Sex (% male)	45	45	65
N	21	20	20

449 Data are shown as mean ± SEM for N observations per group. *ANOVA indicated that, with
450 adjustment for gestational age, birthweight was influenced by maternal BMI (P=0.021)

451 **Table 3** Human placental gene expression

	BMI <25	BMI 25-35	BMI >35
ApoA2	1.04 ± 0.21	1.22 ± 0.27	0.53 ± 0.06
ApoB **	0.34 ± 0.06	2.19 ± 0.55	1.04 ± 0.25
ApoC2	0.90 ± 0.15	1.02 ± 0.25	0.76 ± 0.13
Cubn	1.22 ± 0.21	1.15 ± 0.15	0.86 ± 0.09
Fgg	0.48 ± 0.14	0.82 ± 0.41	0.65 ± 0.42
Gpc3	0.97 ± 0.20	1.30 ± 0.20	0.83 ± 0.19
Mttp	0.57 ± 0.08	1.23 ± 0.37	0.42 ± 0.08
Prf1	1.14 ± 0.19	0.85 ± 0.10	1.03 ± 0.17
Rbp4 **	0.50 ± 0.13	0.07 ± 0.01	0.08 ± 0.01
SerpinG1	1.45 ± 0.24	0.90 ± 0.07	0.90 ± 0.11
Tagln	0.97 ± 0.11	1.04 ± 0.10	1.11 ± 0.10
Tbp	1.02 ± 0.11	1.16 ± 0.11	1.00 ± 0.07
Ttr	1.92 ± 0.90	0.23 ± 0.08	0.56 ± 0.20
Vil1	1.14 ± 0.20	1.82 ± 0.33	1.01 ± 0.18

452 * Indicates a significant effect of maternal BMI on placental gene expression (**P<0.01).

453

Supplementary Table S1 Primer sequences used for the determination of gene expression by RT-qPCR

Target Genes		Rat	Human
Cyclophilin A	FWD:	TGATGGCGAGCCCTTGG	CCCCACCGTGTTCTTCGA
	REV:	TCTGCTGTCTTTGGAACTTTGTC	TGCTGTCTTTGGGACCTTGTC
ApoA2	FWD:	ACTGACTATGGCAAGGATTTGATG	GACCGTGACTGACTATGGCA
	REV:	CTCCTGTGCATTCTGAAAGTAAGC	CAAAGTAAGACTTGGCCTCGG
ApoB	FWD:	-	GGGCAGTGTGATCGCTTCA
	REV:	-	GCGGGTCATGCCTTTGAT
ApoC2	FWD:	GAGCACTTGTTCAGTTACTGGAACTC	GACAGCCGCCCAGAACCT
	REV:	TGCTGTACATGTCCCTCAGTTTCT	TTGCTGTACAAGTCCCTGAGTTTC
Cubn	FWD:	TGCATGTCACCTTCACGTTT	GGCGGATCACCCTAATGTTTAA
	REV:	TGTAAAGCCTCTCCCACTCC	TTGAATACTATCACATGCTCATTGTTG
Fgg	FWD:	CTGGCTGGTGGATGAACAAGT	TCTGGTTGGTGGATGAACAAGT
	REV:	TGGAGTAAGTGCCACCTTGGT	TTTTGAGTAAGTGCCACCTTGGT
Gpc3	FWD:	CGGTTTTCCAAGAGGCCTTT	TCGTGGAGAGATACAGCCAAAA
	REV:	GTAGAGAGACACATCTGTGAAAAATTCA	GGCTCAGGGCCCTTCATT
Mttp	FWD:	TTTTCCTCTGTTTCTTCTCCTCGTA	GAGTGGATCTTCTTCTGCCTACACT
	REV:	AGCTTGTATAGCCGCTCATTATTTAAT	CCAGAACCCGAGTAGAGAATGTCT
Prf1	FWD:	GCTGGCTCCCATTCCAAGAT	CAACTTTGCAGCCCAGAAGAC
	REV:	GCCAGGCGAAAACTGTACATG	TGTGTACCACATGGAAACTGTAGAAG
Rbp4	FWD:	GAGGAAACGATGACCACTGGAT	ACGAGACCGGCCAGATGA
	REV:	TGCAGGCGGCAGGAATA	CACACGTCCCAGTTATTCAAAAGA
SerpinG1	FWD:	GACAGCCTGCCCTCTGACA	GCCCAGACCTGGCCATAAG
	REV:	TTTCTTCCACTTGGCACTCAAG	CCAAGTTGGCGTCACTGTTG
Tagln	FWD:	GGCGTGATTCTGAGCAAGTTG	GGCGTGATTCTGAGCAAGCT
	REV:	CATGGAGGGCGGGTTCTC	GACCATGGAGGGTGGGTTCT
Ttr	FWD:	CCGTTTGCCTCTGGGAAGA	CTTGCTGGACTGGTATTTGTGTCT
	REV:	CCCCTTCCGTGAACTTCTCA	AGAACTTTGACCATCAGAGGACACT
Vil1	FWD:	FWD: AACCAGGCTTTGAACTTCATCAA	CTGAGCGCCCAAGTCAAAG
	REV:	REV: CGGACTCAGCCCCATCATT	CATGGCCTCGATCCTCCATA

Forward (FWD) and reverse (REV) primer sequences designed using Primer Express software (version 1.5; Applied Biosystems)