THE EARLY ORIGINS OF OBESITY: THE IMPORTANCE OF PRENATAL VS POSTNATAL ENVIRONMENT

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DECLARATION

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COMMONLY USED ABBREVIATIONS

A B C

AA	arachidonic acid
ACC	acetyl-CoA Carboxylase
ACS	acyl-CoA synthetase
ACOD	acyl-CoA oxidase
ad libitum	to any desired extent
ADD-1	adipocyte determination and differentiation-1
ALA	alpha-linolenic acid
ANOVA	analysis of variance
ASP	acylation-stimulating protein
ATP	adenosine triphosphate
ATGL	adipose triglyceride lipase
AUC	area under the curve
BAT	brown adipose tissue
BHT	butylated hydroxyl toluene
BMI	body mass index
C	control
CAF	cafeteria
cDNA	complementary deoxyribonucleic acid
cAMP	cyclic adenosine monophosphate
CART	cocaine- and amphetamine-regulated transcript
C/EBPα	CCAAT/enhancer-binding protein-alpha
C/EBPβ	CCAAT/enhancer-binding protein-beta
C/EBPγ	CCAAT/enhancer-binding protein-gamma
CoA	Coenzyme A

D E F G

D	day(s)
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
dsDNA	double stranded deoxyribonucleic acid
DR	diet resistant
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EIA	enzyme immunoassay
EPA	eicosapentaenoic acid

ERRα	estrogen related receptor alpha
FABP	fatty acid binding protein
FAME	fatty acid methyl esters
FATP	fatty acid transport protein
FAS	fatty acid synthase
FFA	free fatty acids
FIAF	fasting–induced adipose factor
FID	flame ionisation detector
GDP	guanosine diphosphate
GDM	gestatational diabetes mellitus
GH	growth hormone
G3PDH	glycerol 3-phosphate dehydrogenase
G6PD	glucose-6-phosphate dehydrogenase
GPAT	glycerol-3-phosphate acyltransferase

H I J K L

HRP	horseradish peroxidase
HSL	hormone sensitive lipase
IGFs	insulin-like growth factors
IGF-I	insulin-like growth factor I
IPGTT	Intraperitoneal glucose tolerance tests
IRS	insulin receptor substrate
LA	linoleic acid
LDL-R	lipoprotein receptor
LPL	lipoprotein lipase
LCPUFA	long chain polyunsaturated fatty acids

M N O

ME	malic enzyme
MEFA	methy-N-ethyl-N(β-hydroxyethyl)-aniline
mRNA	messenger ribonucleic acid
min	minute(s)
MGL	monoglyceride lipase
NEFA	non-esterified free fatty acids
n-3	omega-3
n-6	omega-6

P Q R S

PEPCK	phosphoenolpyruvate carboxykinase
POD	peroxidase
PGAR	PPAR-γ angiopoietin related peptide
PI3K	phosphoinositide 3-kinase
6-PG	6-phosphogluconate
PND1	postnatal day 1
PPAR-γ	peroxisome proliferator- activated receptor gamma
PUFA	polyunsaturated fatty acids
RBC	red blood cells
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
qRT-PCR	quantitative real time PCR
RXR	retinoid-acid receptor
SC	subcutaneous
SCD-1	stearoyl-CoA desaturase-1
SEM	standard error of the mean
SPSS	statistical package for social sciences
SREBP	sterol regulatory element binding proteins

T U V W X Y Z

T2DM	type 2 diabetes mellitus
TG	triglyceride
TGFα	transforming growth factor-alpha
TGFβ	transforming growth factor-beta
TLC	thin layer chromatography
TMB	Tetramethylbenzidine
TNF-α	tumour necrosis factor-α
UCP-1	uncoupling protein 1
WAT	white adipose tissue
WHO	World Health Organization

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RELATED PUBLICATIONS

1. Gugusheff, JR., **Vithayathil, M**., Ong, ZY., & Muhlhausler, B. S. (2013). The effects of prenatal exposure to a 'junk food'diet on offspring food preferences and fat deposition can be mitigated by improved nutrition during lactation. *Journal of Developmental Origins of Health and Disease*, *4*(05), 348-357.

2. Muhlhausler, BS., Gugusheff, JR., Ong, ZY., & **Vithayathil, M. A**. (2013). Pregnancy, obesity and insulin resistance: maternal overnutrition and the target windows of fetal development. *Hormone Molecular Biology and Clinical Investigation*, *15*(1), 25-36.

3. Muhlhausler BS, Gugusheff JR, Ong ZY, **Vithayathil MA**. Nutritional approaches to breaking the intergenerational cycle of obesity. *Canadian Journal of Physiology and Pharmacology.* 2013; 91:421-8.

4. Muhlhausler, B. S, **Vithayathil, M. A.** Imapct of maternal obesity on offspring adipose tissue: lessons for the clinic. Expert Review of *Endocrinology &amp Metabolism* 10/2014; 9(6).

ABSTRACT

There is growing evidence that maternal obesity, maternal hyperglycemia or maternal intake of diets high in fat, sugar or total calories during pregnancy and lactation is associated with an increased risk of obesity and metabolic diseases in the offspring. The majority of studies to date, however, have examined the impact of maternal overnutrition during the entire perinatal period. While a small number of studies have provided clues that the impact of exposure to nutritional excess before birth in comparison to exposure during the early postnatal period may not be equivalent, the results of these studies have been inconsistent. Therefore, the relative contribution of prenatal and postnatal nutritional environment to obesity risk in the offspring remains unclear. The central aim of this thesis was to investigate the separate contributions of exposure to a maternal cafeteria diet during the prenatal and suckling periods on the metabolic outcomes of the offspring, specifically body weight, fat mass and the expression of key adipogenic and lipogenic genes at weaning, in early adolescence and in young adulthood using a cross-fostering approach in a rat model.

The results of this thesis demonstrated that exposure to a maternal cafeteria diet during the suckling period is more important for determining fat mass at weaning than exposure before birth. Importantly, this thesis provided considerable evidence to suggest that exposure to a nutritionally-balanced diet during the suckling period has the capacity to prevent the negative effects of exposure to a high-fat/high-sugar diet before birth. In addition, this thesis has demonstrated that the effects of being exposed to a high-fat/high-sugar diet during the perinatal period on offspring adiposity could be reversed/controlled by consuming a nutritionally-balanced diet post-weaning.

The results of this thesis also demonstrated that the levels of total fat, saturated and trans fats and omega-6 polyunsatured fatty acids (n-6 PUFA) in the dams

milk were directly related to their levels in the maternal diet, and were higher in dams consuming a cafeteria diet. This supported the hypothesis that altered fat content and fatty acid composition of the milk is likely to play an important role in mediating the effects of maternal cafeteria diets on offspring fat mass, and may well account for the higher adiposity at weaning in offspring suckled by cafeteria-diet fed dams. Exposure to a cafeteria diet during the suckling period also resulted in altered expression of key adipogenic and lipogenic genes in visceral and subcutaneous fat depots and an increased susceptibility to diet-induced obesity in females. Importantly, this thesis provided evidence of clear sex-differences in the relative impact of prenatal and postnatal nutritional exposures on adipocyte gene expression and the susceptibility to diet-induced obesity in the offspring, suggesting that the timing of nutritional interventions aimed to re-program the offspring may be different in males and females.

Overall, this thesis identifies the early postnatal period in rodents as a ‘critical window’ for the programming of fat mass and susceptibility to diet-induced obesity in the offspring, and has provided important insights into the mechanisms underlying the early origins of obesity.

CHAPTER 1

CHAPTER 1: LITERATURE REVIEW

1.1 OBESITY: A PUBLIC HEALTH EPIDEMIC

Overweight and obesity occur as a result of an excess accumulation of body fat, which is ultimately the result of caloric intake that exceeds energy usage. Obesity is defined as a body mass index (BMI) of ≥30.0kg/m^2 and overweight as a BMI of 25.0- 29.9kg/m^2 in adults [1]. Obesity is currently a major public health issue across the developed and developing world [2, 3]. There has been significant increase in the prevalence of obesity over the past three to four decades and, according to statistics released by the World Health Organization (WHO), the number of obese adults world-wide in 2008 had increased to twice the rate that it was in 1980 [4]. At least 1.4 billion adults were overweight or obese in 2008, with a higher prevalence in females than males [4]. Overweight and obesity are associated with an increased risk of serious health consequences, most importantly type 2 diabetes mellitus (T2DM) and cardiovascular diseases [5]. Overweight and obese women are also at greater risk of hormone-dependent cancers, including cervical, endometrial, ovarian and post-menopausal breast cancer [6]. Obesity and its associated co-morbidities represent a significant burden on healthcare systems and economies of developed and developing nations across the globe [2, 3].

1.1.1 Childhood obesity

Globally, at least 40 million children under the age of 5 years were overweight or obese in the year 2011 [7]. Childhood obesity is increasingly being recognised as a serious public health concern because of its adverse health effects and the significant increase in its incidence over the past three decades. Obese children are at increased risk of a range of associated health problems, including T2DM, high blood pressure, heart disease and sleep disorders [8]. Overweight and

obese children and adolescents are also more likely to become obese adults [9], and previous studies have found that almost 80% of children who were overweight at 10 to 15 years were obese by the age of 25 years [10]. Childhood overweight and obesity represents the outcome of a complex interaction of many variables including genetic, behavioural, environmental, and socio-demographic factors [9]. A number of lifestyle factors also contribute to weight gain, including poor nutrition and reduced physical activity (sedentary lifestyle). However, even when all of these contributing factors are taken into account, it has been shown that there is still a positive relationship between prenatal/perinatal exposure to maternal obesity, maternal diabetes, and/or maternal overnutrition and the risk of obesity in child and adult life [11-14].

A wide range of epidemiological studies have supported a role of the intrauterine environment in programming the metabolic health of individuals through the life course. The early origins of obesity and poor metabolic health have been further supported by the results of experimental animal studies, which have shown that exposure of the fetus to an 'obeseogenic' intrauterine environment, in which nutrient supply is elevated, results in permanent alterations in the pathways controlling appetite, metabolism and activity levels in the offspring, thus predisposing them to an increased risk of weight gain and excess fat deposition after birth [15-17].

1.1.2 Obesity in pregnancy

The dramatic rise in incidence of obesity in the population has led to a corresponding increase in the number of women entering pregnancy either overweight or obese [18, 19]. Pregnancy Risk Assessment Monitoring System data from 9 States of United States released in 2007 reported a greater than 69% increase in the pre-pregnancy obesity rate between the years 1992/93 and 2002/03 [18]. Data from the South Australian Pregnancy Outcome Database also reported that more than 50% of pregnant women in South Australia in the year 2008 were overweight or obese when they attended their first antenatal appointment [20]. Maternal obesity is associated with an increased risk of

pregnancy complications including pre-eclampsia, gestational diabetes mellitus (GDM) and caesarean delivery. It is also associated with an increased risk of delivering a macrosomic or large for gestational age infant, who is in turn at an increased risk of later childhood obesity and its associated co-morbidities [21]. Thus an intergenerational cycle of obesity has been proposed wherein overweight and obese mothers give birth to heavy infants who go on to be overweight/obese as children and adults [22]. It has been suggested that the link between maternal obesity and increased risk of obesity in the offspring is due to exposure of the fetus/neonate to an increased supply of nutrients during critical windows of development, which leads to altered development of the systems which regulate food intake and fat deposition and permanent changes in the way these systems function throughout life [15-17].

1.1.3 Maternal obesity, high birth weight and obesity in later life

A large number of epidemiological studies have provided compelling evidence that maternal obesity or increased maternal body mass before and during pregnancy has a role in the programming of child and adult obesity. Results from one of the largest longitudinal cohort studies conducted in Britain showed that both male and female babies with the highest birth weights had the highest adult BMI at 33 years of age. Importantly this relationship was explained largely by the presence of high pre-pregnancy weight and BMI in the mother [23]. These observations have been supported by the results of later studies from Jerusalem, [24] United Kingdom, [25] Northern Finland [26, 27] and Sweden [28] highlighting the fact that maternal overweight and obesity before and during pregnancy is likely to result in a heavy infant that is more likely to be overweight or obese in child and adult life. Neonates of overweight and obese mothers also have a higher body fat mass at birth [29, 30] and a positive relationship has been reported between body fat mass of neonates at birth and body fat mass of obese mothers [31]. It has also been demonstrated in rodent studies that increased body weight among offspring of obese and overfed mothers is accompanied by increased fat mass, fat cell hypertrophy, reduced muscle mass, higher glucose, insulin, leptin and triglyceride levels and elevated blood pressure

as a result of altered cardiovascular structure and function [16, 17, 32]. These studies therefore suggest that maternal obesity and childhood obesity are causally linked, thus setting up an intergenerational cycle of obesity and associated poor metabolic health (Figure 1.1).

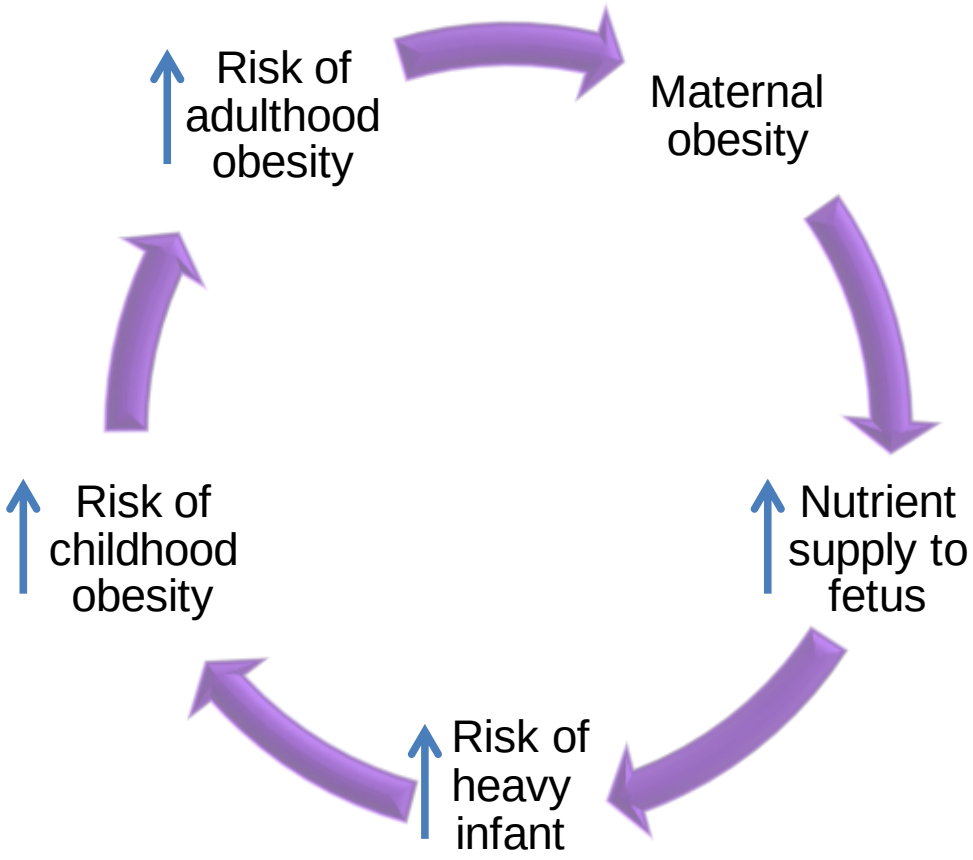


Figure 1.1 Schematic representation of the intergenerational cycle of obesity. Adapted from McMillen I C et.al. [22].

1.2 DEVELOPMENTAL PROGRAMMING OF OBESITY

1.2.1 The developmental origins of health and disease hypothesis

The process by which exposures during the perinatal period can be causally linked to later consequences for health and disease is referred to as developmental programming. Lucas first defined this process as "either the induction, deletion, or impaired development of a permanent somatic structure or the 'setting' of a physiological system by an early stimulus or insult operating at a 'sensitive' period, resulting in long term consequences for function" [33]. It is becoming increasingly evident that exposure to a perturbed nutritional or metabolic environment during critical periods of development can have long-term effects on the risk of obesity in adulthood [34-36]. 'The fetal origins hypothesis' was first proposed by Barker and colleagues in the early 1990s, and led to a series of studies, which have shown that there is a J or U-shaped relationship between birth weight and adult obesity, hypertension [37], insulin resistance [38], vascular dysfunction [39] and dyslipidemia [40] with increases in risk occurring at both the low and high end of the birth weight spectrum.

1.2.2 The role of nutrition in developmental programming

One critical factor for fetal health and survival is the supply of nutrients and oxygen from the mother. A mother's ability to provide nutrients for her baby in turn depends on her nutritional status, metabolism, body composition and body size, all of which develop throughout the mother's own fetal life, childhood and adolescence [41]. The fetus has a poor ability to respond to changes in nutrient supply by altering nutrient intake since it obtains its nutrition from transplacental transfer from maternal circulation [42], and is thus particularly susceptible to changes in the nutritional environment before birth.

The role of maternal nutrition in the programming of obesity has been studied widely in both human populations and in animal models. The focus of early

studies in the field of developmental programming was on the effect of undernutrition, and the relationship between a low weight at birth and the risk of cardiovascular disease later in life [43]. In 1992, Hales and Barker proposed 'The Thrifty Phenotype' hypothesis, which described the concept whereby poor nutrition *in utero* induces fetal adaptations that produce permanent changes in the structure and function of key physiological systems. These changes, in turn increase the risk of disease in the offspring later in life, including the risk of visceral obesity, T2DM and cardiovascular disease in adult life [44, 45] (Figure 1.2). It has been reported previously that perturbations to the normal intrauterine environment can be detrimental to fetal growth. Failure to supply an adequate amount of nutrients to meet fetal demand as a result of maternal malnutrition, inadequate placental function or increased nutritional demand (excessive energy utilisation), results in an inadequate supply of nutrients to the developing fetus [41, 46, 47]. In the case of prolonged undernutrition, the fetus changes its metabolic rate and alters the production of hormones and sensitivity of tissues to them [47, 48]. Decreased maternal food intake leads to a fall in the concentrations of fetal glucose and insulin and ultimately reduces the rate of fetal growth [49] (Figure 1.2). The 'Predictive Adaptive Response' hypothesis [50-52] proposed later by Gluckman and Hanson proposes that the fetus will make adaptations *in utero* or in the early postnatal period based on the predicted postnatal environment. According to this hypothesis, when this predictive adaptive response is matched to the postnatal environment, the phenotype of the offspring will be normal. However, where the actual environment exceeds the predicted environment, this adaptation places the individual at increased risk of diseases, in particular obesity and associated metabolic diseases (Figure 1.2). However, neither of these hypotheses provided a clear mechanism to explain the alterations in the adult offspring following maternal overnutrition.

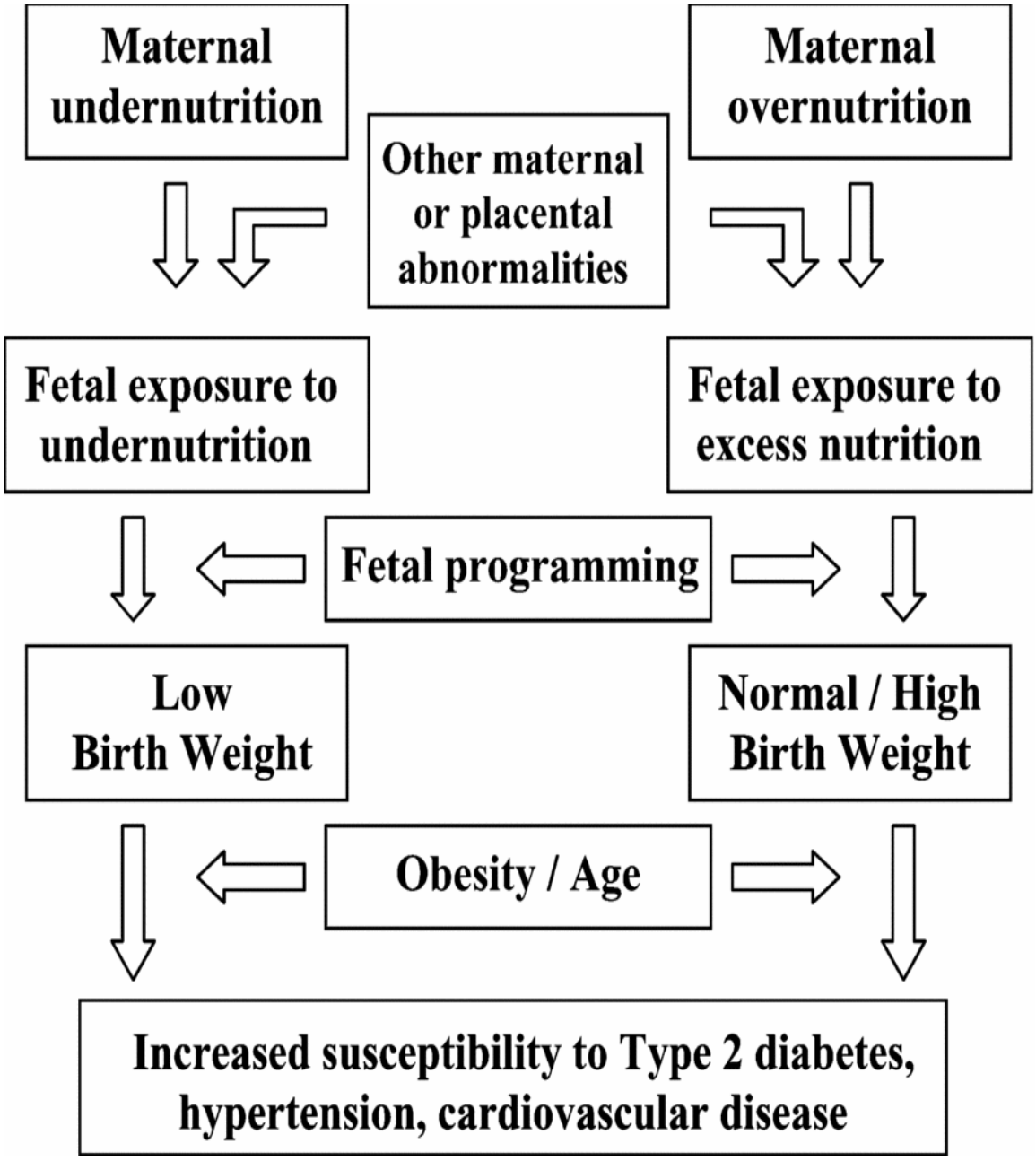


Figure 1.2 Schematic representation of the effects of maternal nutrition on the health of the offspring. From Martin-Gronert et al. 2006 [41].

1.2.2.1 *Epidemiological studies*

The earliest epidemiological studies examining the effects of poor maternal nutrition and its effect on birth weight and subsequent adult health and disease were based on a study of adult individuals whose mothers had been pregnant during the 'The Dutch Hunger Winter' [53, 54]. During the last six months of World War II, from October 1944 until liberation on May 7 1945, an acute famine which was worsened by an unusually early and hard winter, affected The Netherlands. This Dutch famine affected people of all social classes and was followed by growing prosperity in the post-war period. The results from the Dutch Hunger Winter study were first published in 1976 and provided an almost flawlessly designed, although tragic, human experiment in the effects of intrauterine scarcity on subsequent adult health. Increased adult obesity and glucose intolerance was reported in babies exposed to the Dutch Famine during the late gestation period, and hypertension during adulthood was reported in babies exposed to famine during the early gestation period [53, 55]. The relationship between poor *in utero* nutrition and an increased risk of cardiovascular and metabolic diseases in the offspring later in life has also been reported in a range of studies in other disadvantaged populations [56-59].

The link between maternal overnutrition and detrimental health outcomes in the offspring has now also been investigated in a large number of population studies. The studies of infants of diabetic mothers led Freinkel [60] to hypothesise that increased maternal nutrition may alter the normal development of the fetus. He suggested that an elevated supply of fuels to the developing fetus could result in permanent changes in endocrine or neuroendocrine metabolism. Thus it was suggested that factors associated with the diabetic environment *in utero*, e.g., exposure to elevated concentrations of glucose, amino acids, lipids, ketones, and possibly altered concentrations of other nutrients, may have a direct effect on the fetus, increasing insulin secretion and potentially leading to the development of resistance to insulin-mediated glucose disposal in postnatal life [60]. It has also been reported in a study in Pima Indians that children born to women with GDM were more susceptible to the

development of insulin resistance in postnatal life [60, 61]. Studies of infants born to women who had diabetes during their pregnancy also provided the first compelling evidence of a link between increased maternal and hence fetal nutrition and increased fat mass and adverse metabolic health outcomes in the offspring [62-65]. These studies showed that increased maternal glucose concentrations, as a result of maternal diabetes or impaired maternal glucose tolerance, resulted in fetal hyperglycemia, fetal overgrowth and increased fat deposition *in utero* [62-65]. It was also subsequently shown that these individuals were at increased risk of obesity and T2DM in child and adult life [62-65]. It has also been suggested that women who develop diabetes before or in the early stages of pregnancy may transmit increased genetic susceptibility to their offspring, and that this may explain the increased susceptibility towards later development of T2DM in these individuals [66]. However, evidence from previous studies suggests that genetic factors are not the only factor which contributes to the excess growth experienced by offspring of diabetic mothers, because obesity and T2DM is less common in the offspring of women who became diabetic after delivery [67, 68] and the offspring of diabetic fathers do not show the excessive prenatal growth and increased obesity risk observed in the offspring of diabetic mothers in studies of the Pima Indians [69, 70]. Thus, epidemiological studies clearly demonstrate that the increased risk of abnormal glucose homeostasis in offspring is associated with intrauterine exposure to diabetes beyond that attributable to genetic factors [66] (Figure 1.3). Overall, therefore the results from these epidemiological studies provided evidence for a link between maternal overnutrition and the risk of obesity in the offspring later in life and suggested that exposure to an increased nutrient supply during the perinatal period programs an increased susceptibility to developing obesity and T2DM in later life.

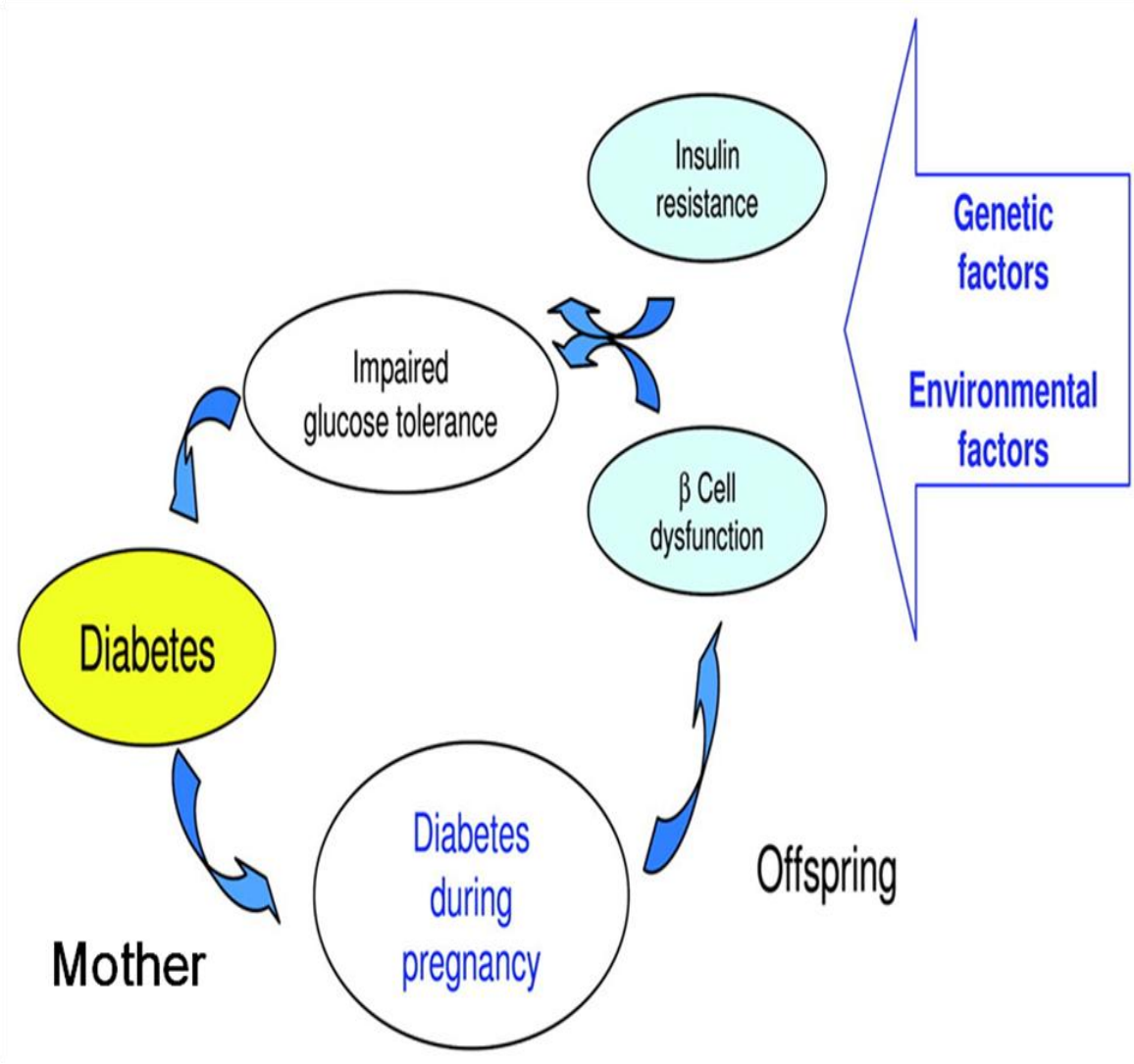


Figure 1.3 The proposed pathway for the intergenerational transmission of T2DM. Adapted from Lila Sabrina Fetita et.al. [66].

1.2.2.2 *Sheep models*

The sheep is widely used as a large animal model of developmental programming due to its relatively similar developmental profile and maturity at birth to human infants [71]. The majority of studies in sheep have focussed on the short-term effects of suboptimal intrauterine environments on the fetus rather than longer term outcomes [72-74]. However, a number of studies have demonstrated that nutrient restriction or maternal undernutrition from early to mid gestation leads to growth retardation, cardio-ventricular hypertrophy and altered development of key metabolic systems [73, 75]. More recently, studies in the sheep model have also provided evidence that increased maternal nutrient intake during gestation results in higher plasma glucose levels in the lamb in the immediate postnatal period and higher subcutaneous fat mass at 30 days of age [76]. Other studies have reported that the adverse metabolic effects of maternal overnutrition in sheep also persisted into adulthood resulting in altered growth, relative hyperphagia (excess food intake as a proportion of their current body weight) and increased adiposity in the adult offspring [77]. It has been also reported that lambs of over-nourished ewes exhibited impaired insulin sensitivity, glucose homeostasis and higher plasma leptin levels in adulthood [78].

1.2.2.3 *Rodent models*

Rats and mice are the most commonly used animal models for the investigation of developmental programming via maternal undernutrition or overnutrition [79-81]. This is mainly because the shorter life span and gestational period of rodents, which allows the effects of *in utero* exposures on long-term outcomes in the adult offspring to be studied over a relatively short time frame. In addition, genetic and environmental variables can be controlled more easily in rodents than in humans and large animal models [82]. The results of these studies have also strongly supported the epidemiological evidence discussed above. It has been reported in rats that impaired intrauterine nutrition due to maternal protein restriction during gestation results in offspring with low birth weight who go on to

develop impairments in glucose tolerance with age as a result of reduced beta cell mass, reduced insulin secretion and insulin resistance in peripheral tissues [83-87]. Total caloric restriction during pregnancy in rats has also been reported to result in hyperphagia and hyperinsulinemia in the offspring, and these offspring also have a higher fat mass and develop hypertension later in life [88].

Rodent studies of maternal overnutrition have also shown that feeding pregnant and lactating dams either a cafeteria or high-fat diet results in obesity, insulin and leptin resistance and hypertension in their offspring [89-91]. Even mild to moderate maternal overnutrition has been reported to induce increased adiposity, glucose intolerance, hyperinsulinemia in male and female offspring [92, 93]. It has also been reported that maternal feeding on a high-fat or cholesterol-rich diet during pregnancy and lactation results in an offspring phenotype which resembles human metabolic syndrome [8].

1.2.3 Programming of obesity by maternal overnutrition: the proposed mechanisms

A number of theories have been put forward to explain the link between maternal and fetal overnutrition and the increased risk of obesity in the child (Figure 1.4). The mechanisms through which this occurs are still not completely understood. However, it is generally accepted that an increase in maternal glucose concentrations results in an increase in fetal glucose concentrations, which stimulates insulin release from the fetal pancreas, resulting in fetal hyperinsulinemia [94] and that this in turn, can promote excess fat deposition and can induce insulin and leptin resistance in part by down-regulation of insulin and leptin receptors [95]. Programmed central insulin and leptin resistance can then result in hyperphagia which further increases the risk of obesity in postnatal life [95-97]. Elevated maternal and fetal glucose concentrations and fetal hyperinsulinemia and hyperglycemia throughout gestation has been reported previously in pregnancies complicated by maternal glucose intolerance [94]. Importantly, It has been reported that these infants demonstrate increased body weight, adiposity and elevated leptin concentrations at birth [98] and increased

risk of obesity in later life [5, 99, 100]. It therefore appears that exposure to high substrate concentrations in late gestation is associated with an increased risk of obesity in the child.

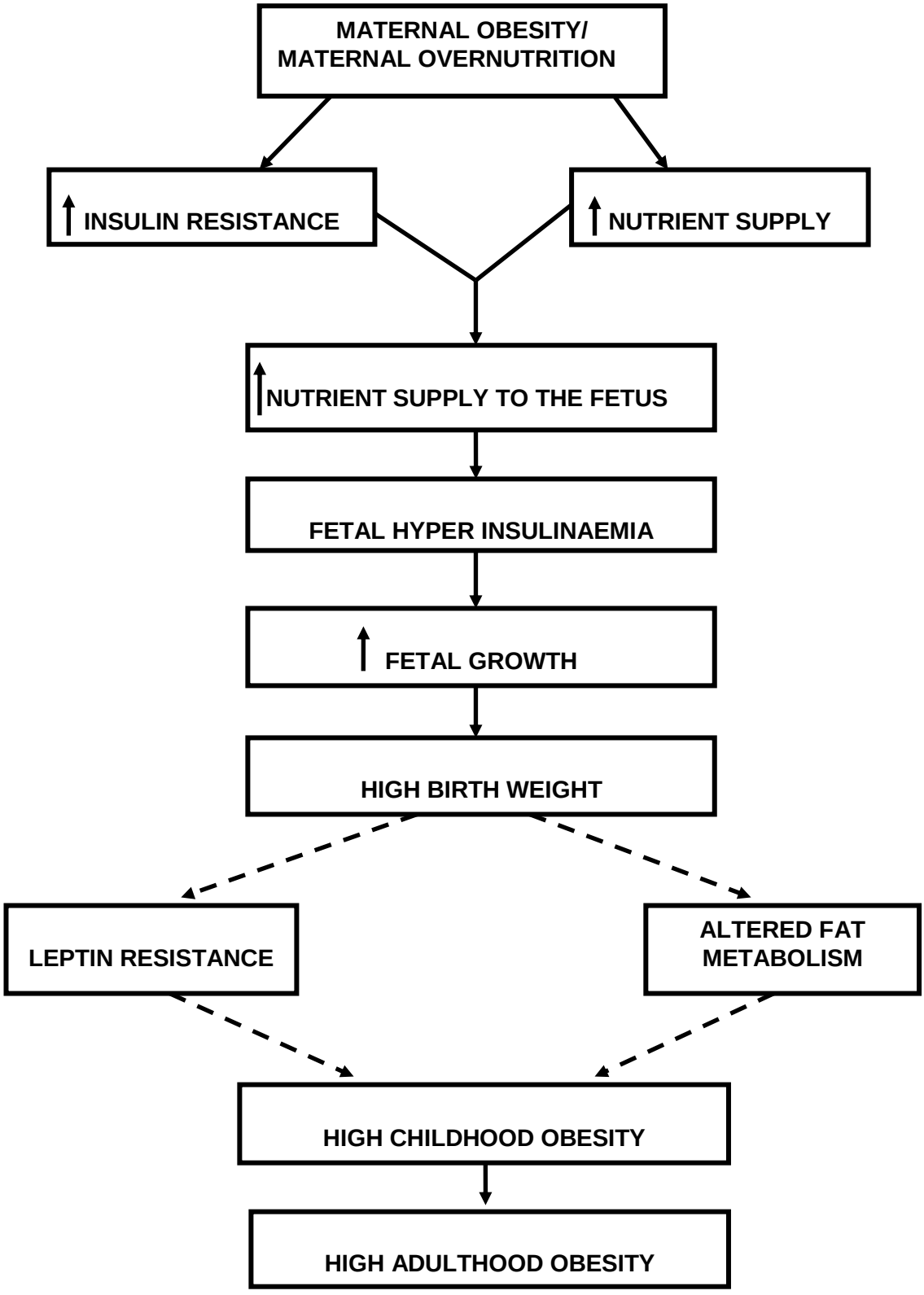


Figure 1.4 A summary of the potential mechanisms which have been proposed to underlie the development of obesity after exposure to maternal nutrition or maternal obesity before birth.

1.2.4 Prenatal/ perinatal programming of postnatal obesity

A number of studies have demonstrated that the development of several key metabolic systems, including the appetite regulating network, fat cells and systems controlling insulin signalling and metabolic rate is strongly related to the nutritional conditions to which the fetus/neonate is exposed during the perinatal period [34-36]. For example, Muhlhausler and colleagues [76] demonstrated using a sheep model that the exposure to maternal overnutrition (which increased glucose and leptin concentrations in the maternal circulation) *in utero* resulted in alterations to appetite regulation in the lamb in early postnatal life. This study showed that in the first 3 weeks of postnatal life, lambs of over-fed ewes exhibited relative hyperphagia together with an increase in plasma glucose concentrations compared to lambs of control ewes [76]. The rat studies on high-fat feeding conducted by Howie and colleagues demonstrated a significantly increased risk of obesity with associated hyperinsulinaemia and hyperleptinaemia in both male and female adult offspring, as a result of maternal high-fat consumption during pregnancy and lactation, independent of the post weaning diet [93, 101]. It has also been reported in rodent studies that maternal high-fat diets alter glucose homeostasis [13] in the offspring and also leads to the development of hypertension [102], abnormal serum lipid profiles [13, 102], endothelial dysfunction [102], increased adiposity [13, 103] and hyperleptinaemia [41, 104, 105] (Table 1.1). Thus it is clear from the previous studies that maternal overnutrition and gestational diabetes have undesirable effects on the developing fetus that cause neonatal morbidities and increased risk of obesity and T2DM later in life.

Table 1.1 Metabolic disorders and diseases of adulthood that have been associated with nutritional imbalances during fetal life. Adapted from Martin-Gronert et al. 2006 [41].

Metabolic disorders	β-Cell dysfunction, Dyslipidaemia, Glucose intolerance, Insulin resistance, Obesity, Type 2 diabetes
Cardiovascular disorders	Hypertension, Atherosclerosis, Stroke, Coronary heart disease
Osteoporosis	
Cancer	Breast cancer
Chronic obstructive lung disease	
Chronic renal failure	
Polycystic ovary syndrome	
Psychiatric disorders	Schizophrenia

1.2.5 The role of early postnatal environment in the programming of obesity

Although it is clear from existing studies that the prenatal environment is a major determinant in the programming of obesity, there is growing evidence that the early postnatal environment also has an important role in the regulation of the development of the systems which regulate fat deposition, energy balance and neonatal ingestive behaviour and also has the potential to affect the long-term metabolic health of the offspring [106-109]. Since developmental programming is not limited to the effects of prenatal exposures and the rapid growth and development of physiological systems continues after birth, the maternal nutritional environment during the postnatal period is also likely to have a role in determining the susceptibility to obesity and T2DM in the offspring. There is growing interest in understanding the relative contribution of prenatal and postnatal nutritional environments to the developmental programming of increased obesity and T2DM risk in the offspring; however the relative contribution of these two periods remains unclear, and this forms the major focus of this thesis.

Cross-fostering approaches have been used previously in rodent models to investigate the relative importance of prenatal and postnatal maternal nutrition on growth, body weight and body fat mass in the offspring [12, 106, 107, 110-115]. However, these studies have produced inconsistent results. Some studies have reported that the prenatal maternal nutrition accounted for a greater proportion of the variance in offspring body weight than postnatal effects [12, 115]. Other studies, however, have suggested that postnatal exposures during the suckling period were more important in determining body weight in the offspring at and after weaning [107, 108].

A cross-fostering study conducted by Chang and colleagues [116] demonstrated that exposure to a maternal high-fat diet before birth alone resulted in higher body weight and increased deposition of fat mass in the offspring, implicating the fetal period as a critical time in programming of obesity. However, the results

of a similar study by Gorski and colleagues led to the opposite conclusions. These researchers found that cross fostering offspring of diet-resistant (DR) dams to obese dams during lactation resulted in obesity and insulin resistance in the offspring, suggesting that exposure to an 'obesogenic' environment during the suckling period could result in an increased risk of obesity in the offspring, even if they had been born to lean, obesity resistant dams. Conversely, in this same study, offspring of obese dams cross-fostered onto DR dams had improved insulin sensitivity compared to those cross-fostered onto another obese dam, again supporting the importance of the nutritional environment during suckling period for the metabolic programming of the offspring [107]. Bayol and colleagues also identified the suckling period as a critical period for metabolic programming, and demonstrated that exposure to maternal "junk food" feeding during the lactation period was more important in increasing the susceptibility of the offspring to hyperphagia and obesity later in life than the same "junk food" diet during pregnancy [117]. Khan and colleagues also reported that exposure to a maternal lard–rich diet during the suckling period alone resulted in elevated blood pressure, depressed endothelial function and the development of abnormal glucose homeostasis in the adult offspring, supporting the important role of nutritional exposures during the suckling period for the programming of cardio metabolic health outcomes [102].

While these and other studies have provided evidence in support of the importance of postnatal period in the development of obesity and other metabolic disorders in the offspring, the biological mechanisms involved are largely unknown. However, given that the breast-milk provides the sole source of nutrients to the breast-fed offspring during the suckling period, alterations in breast-milk composition are likely to play a key role.

1.2.5.1 Protective effect of breastfeeding against obesity?

Breastfeeding is recommended as the gold standard for infant feeding as it provides optimal nutritional, immunological, and emotional nurturing [118-120]. No other milk supplement is the same as breast-milk in terms of growth factors,

nutrients, enzymes, hormones, anti-inflammatory and immunological properties [119, 120]. Breast-milk also contains a full complement of all essential polyunsaturated fatty acids (PUFA) [121]. It has been demonstrated that the growth kinetics of breast-fed infants differ from formula-fed infants as formula-fed infants show higher weight and length gains in early infancy [122].

The associations between breastfeeding and child obesity has been debated for many years and there was initially inadequate evidence to draw any clear conclusions. However, recent studies suggest that breastfeeding reduces the risk of child obesity to a moderate extent [123, 124]. Several population-based studies suggested that breastfeeding was protective against the development of obesity in the child [125, 126]. In Pima Indians, a population which has been identified as having a particular predisposition to obesity and T2DM, individuals who were breast-fed for at least two months had lower weight for height and lower rates of T2DM at 10-39 years of age compared to those exclusively bottle-fed [127]. A lower risk of T2DM has also been reported in Native Canadian children who were breast-fed for at least 12 months compared to those breast-fed for <12months [128]. The protective effect of breastfeeding against the later development of obesity in the child was also reported in a meta-analysis, published in 2005, which included an analysis of 61 studies including almost 300,000 subjects [129]. These results indicated that there was a consistent link between being breast-fed and the duration of breastfeeding and a reduced incidence of obesity, insulin resistance and T2DM later in life.

The potential mechanisms underlying the protective effect of breastfeeding against obesity are based on the unique composition of breast-milk, metabolic and physiologic responses to breast-milk, and the suckling experience [130]. Some studies have suggested that bottle-feeding can impair the development of appetite regulation, because of the tendency to encourage infants to “empty the bottle,” which results in these infants having an impaired ability to recognise satiety cues [131]. It has been also found that children who had highest consumption of breast-milk in early life exhibited the lowest ratio of leptin concentration to fat mass in adolescence suggesting that breastfeeding may

improve leptin sensitivity later in life [132]. It has been reported previously that human milk also contains bioactive substances which influence adipocyte differentiation and proliferation, in addition to its distinctive nutrient composition [133-135]. It has been also reported that the energy intake and protein intake is lower in breast-fed infants compared to formula-fed infants and that this may be important in protecting them from developing obesity later in life [136, 137].

More recent studies, however, have suggested that the relationship between breastfeeding and obesity may not be as robust as originally thought. Kramer et al.[138] from Promotion of Breastfeeding Intervention Trial (PROBIT) Study Group reported that a breastfeeding promotion intervention resulted in substantial increases in the duration and exclusivity of breastfeeding, yet there was no association between breastfeeding duration or exclusivity and the incidence of overweight/obesity in the children at 6.5 years of age [138]. This may be related in part to the changes in the composition of infant formulas such that they more closely resemble the composition of breast-milk. In addition, the composition of breast-milk has also shifted considerably over the past few decades due to changes in the composition in the typical western diet [139-141]. Therefore, it is likely that the role of breastfeeding in the early life origins of obesity is dependent on breast-milk composition, which in turn is dependent on the composition of the maternal diet.

1.2.5.2 The effect of maternal high-fat feeding on breast-milk composition

Maternal diet is a key determinant of breast-milk composition and therefore the nutritional composition of the diet of breast-fed infants [142]. Whilst data are currently limited, there is some evidence that the macronutrient composition of the breast-milk is also influenced by the presence of maternal metabolic diseases, such as diabetes, during the lactation period [143, 144]. The macronutrient composition and energy content of breast-milk from diabetic mothers has been characterised in a small number of studies in humans and it has been shown that the energy content as well as the concentrations of insulin

and glucose are significantly higher in the breast-milk of diabetic mothers compared with non-diabetic mothers [143, 144]. Plagemann and his colleagues also observed in a clinical study that the early neonatal ingestion of breast-milk provided by diabetic mothers was associated with a higher relative body weight and increased prevalence of obesity in the offspring at 2 years of age compared to infants of diabetic mothers who were fed with banked breast-milk from non-diabetic mothers [145]. Similarly, in a rodent study, Fahrenkrog and colleagues demonstrated that neonatal exposure to milk from diabetic mothers resulted in altered development of the systems regulating energy balance and appetite in the offspring and thus contributed to the development of hyperphagia and obesity in postnatal life [146]. Together, these studies suggest that maternal metabolic diseases, in particular diabetes, may influence the breast-milk composition and may have deleterious effect on offspring.

It has also been reported that an increased milk fat content is observed in milk from dams consuming a high-fat diet [147-149] and that the fat content of the milk reflects the fat intake of the mother much more closely than the protein content reflects maternal protein intake [148, 150]. Studies in humans have provided evidence that 30% of individual fatty acids in human milk are derived directly from the maternal diet [151, 152] and the remaining 70% from body stores and liver metabolism [153]. It has also been suggested that the major dietary factors contributing to the variability of the levels of fatty acid composition in human milk are the amounts of carbohydrate and of PUFA and trans fatty acids in the maternal diet [109, 154-158]. One previous study also reported that a maternal high-fat diet can alter the fatty acid composition of the milk, with an increase in the long chain fatty acids at the expense of medium chain fatty acids [159]. This suggests that high-fat diets suppress of the synthesis and secretion of medium chain fatty acids into the breast-milk, which may be important in controlling fat deposition in the growing pup [159]. Together, these studies have suggested that breastfeeding may not always be protective against the later development of obesity, and may even have a negative effect.

1.3 CURRENT OBESITY EPIDEMIC: THE ROLE OF CHANGES IN THE COMPOSITION OF MATERNAL DIET

Shifts in the composition of the typical Western diet over the past 20-30 years have been implicated as a key contributing factor to the current epidemic of obesity and T2DM [160-163]. Comparisons between Paleolithic nutrition and modern Western diets have suggested a substantial shift in dietary composition across this time. In terms of macronutrient composition, there has been a shift towards carbohydrates, in particular refined carbohydrates with a high glycemic load, at the expense of protein [164, 165]. The intake of many key micronutrients (eg: folate, magnesium, potassium and zinc) and dietary fibre has also declined over time, while the intake of sodium has increased [166-168].

Perhaps the most significant changes which has occurred in the composition of the typical Western diet has been a shift in its fat composition, particularly the balance of omega-3 (n-3) and omega-6 (n-6) PUFA, and the majority of these changes have occurred relatively recently (in the past 40 to 50 years). Prehistoric humans evolved on a diet that consisted primarily of fresh fruits, leafy vegetables and animals and all these foods provided a fairly good balance of n-6 and n-3 PUFA upon which physiological and metabolic processes were established. For millions of years the approximate 1: 1 ratio of n-6 to n-3 PUFA remained unchanged [169-171]. Significant changes in the composition of the food supply of Western societies over the past 50 years have resulted in a substantial increase in the consumption of n-6 PUFA while the intake of n-3 PUFA has remained relatively constant [169-172]. As a result, the ratio of n-6 PUFA to n-3 PUFA in the typical Western diet has shifted from 1-2:1 to ≈ 10-20:1. Because of the decrease in fish consumption, the intake of the n-3 long chain fatty acids (LCPUFA) has also declined across this time [167, 173, 174]. It has been also reported that the levels of saturated and trans fatty acids in the modern Western diet are substantially higher than those in traditional human diets [174-177].

1.3.1 The n-3 and n-6 fats: physiological roles

Alpha-linolenic acid (ALA; 18:3n-3, precursor to n-3 series PUFA) and linoleic acid (LA; 18:2n-6, precursor to n-6 series PUFA) are the parent n-3 and n-6 PUFA, respectively. ALA and LA are "essential fatty acids," meaning that the human body is unable to produce them and they can only be obtained from the diet [167, 178]. Nutritionally significant n-3 PUFA include ALA, eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) and these fats are essential for normal growth and cell function, while LA and arachidonic acid (AA; 20:4n-6) are physiologically significant n−6 PUFA. N-3 and n-6 PUFA are precursors to potent lipid mediator signalling molecules, termed “eicosanoids” (eg; prostaglandins, thromboxanes and leukotrienes). Eicosanoids derived from n-6 PUFA are pro-inflammatory [179, 180] while eicosanoids derived from n-3 PUFA are either less potent inflammatory agents or are anti-inflammatory [181]. The pro-adipogenic effect of n-6 PUFA [182] and protective effect of n-3 LCPUFA against obesity [183, 184] and insulin sensitivity [183, 185, 186] are also well documented. As a result, the increased amount of n-6 PUFA relative to n-3 PUFA in modern human diets act to limit the favourable anti-inflammatory and metabolic effects of n-3 PUFA and facilitate the accumulation of adipose tissue, and this has been put forward as a potential factor contributing to the increased incidence of non-communicable diseases in modern society, including obesity and T2DM [167, 187, 188].

1.3.2 The effect of maternal cafeteria/Western diets during lactation on proximate and fatty acid composition of breast-milk

As indicated above, previous studies have demonstrated that the proximate and fatty acid composition of breast-milk is affected markedly by the type of diet available during lactation [159, 189]. However, there are contradictory results from previous rodent studies regarding the effect of the fat content of the maternal diet on breast-milk fat content; with some of the studies reporting that incremental changes in the proportion of fat in the maternal diet during lactation increase the lipid concentration in breast-milk [190], while others reported a

decrease [191] or no effect [192-194]. The milk produced by rats fed on cafeteria diets during lactation has been reported to have a significantly higher energy and fat content and significantly lower protein and lactose concentration compared to those rats fed a standard chow diet [195].

There are currently no studies which have comprehensively assessed the impact of consumption of a cafeteria diet during pregnancy and lactation on the fatty acid composition of the breast-milk. However a number of studies have provided evidence to support shifts in fat composition, as well as fat content, in the milk of humans consuming diets with markedly different fat compositions and rats fed on cafeteria diets during lactation [195, 196]. Comparative studies of the effects of Western diets in Europe and traditional diets in Africa on the fatty acid composition of human milk reported that the amount of saturated fat and monounsaturated fat and n-6 LA in the breast-milk were closely related to their levels in the habitual diet of the lactating women [197]. In contrast, the milk content of 20 and 22 carbon LCPUFA in these studies showed limited relationship to the habitual diet in different geographic regions, with the exception of relatively high n-3 LCPUFA (predominantly DHA) in the milk of African women consuming a large proportion of dietary fat from fish [197]. High amounts of saturated fatty acids in the human breast-milk also have been reported in studies of women from Brazil [198] and Spain [199] who were consuming Western diets high in saturated fats. However, these studies did not find any correlations between dietary fat and milk PUFA, except for 20:5n-3 which correlated positively with dietary saturated fatty acids [198]. Previous studies have also reported that n-3 PUFA in the diet are directly related to n-3 PUFA levels in the breast-milk in humans [200]. An epidemiological study conducted by Ratnayake and Chen reported a negative correlation between the contents of trans fatty acid and LA and ALA PUFA in human milk, suggesting that the rise in trans fatty acids might have a negative influence on the secretion of n-3 PUFA into the milk supply [201]. However, another study reported that the level of LA in human milk was increased by a high trans fatty acid Western diet and there is a direct dose relationship between the intake of trans fatty acids and the incorporation of this PUFA into maternal milk [202, 203]. A growing number

of human and animal studies also support the hypothesis that the changes in the composition of the Western diet with increasing amount of n-6 compared to n-3 PUFA and the resulting shift in n-6/n-3 ratio in the breast-milk of lactating women, may be contributing to the current obesity epidemic [139-141, 204-207].

1.3.3 The role of dietary fat types as early determinants of adiposity in the offspring

The increased incidence of overweight and obesity has led to renewed interest in better understanding the role of the maternal diet during pregnancy and lactation, in particular the level and type of dietary fats as contributing factors to the current epidemic of childhood obesity. Several population-based studies have investigated the relationship between dietary fat intake and body fat in infants and children [208-219], however most of these studies failed to find any association between fat intake during infancy and later adiposity. A review which characterised the role of dietary fats on obesity suggested that the high energy content of a maternal high-fat diet, rather than the fat content *per se,* was likely to be responsible for the hyperphagia and increased adiposity in the offspring [220]. However several previous studies have demonstrated that a maternal high-fat diets can increase the body fat in the offspring, even when there is no accompanying increase in maternal energy intake [221, 222].

To date, little attention has been given to understanding the relative importance of dietary PUFA and LCPUFA for fetal and infant fat deposition, especially the role of n-6 and n-3 PUFAs on early development of adipose tissue and their potential impact on childhood obesity. It has been reported previously that n-6 PUFA have a pro-adipogenic effect [182] and experimental animal studies have reported that increased maternal consumption of LA during pregnancy and lactation results in accelerated maturation of fat depots and increased body fat deposition in the offspring [223]. A recent review on PUFA and childhood obesity presented evidence from animal and human studies that the increased consumption of n-6 PUFAs, in association with a high n-6/n-3 ratio, may promote the development of adipose tissue during the gestation/lactation period

and during infancy [139]. However, whether this is a causal relationship remains to be demonstrated, as other animal studies investigating the role of n-6 and n-3 PUFA in the maternal diet did not find an increase in fat mass in the offspring of dams consuming a high n-6 PUFA diet [206, 224]. An attempt to understand the effect of higher maternal intake of trans fatty acids during pregnancy and lactation on adiposity in rat offspring has also reported that exposure to higher amounts of trans fatty acids during the perinatal period resulted in a long-term alterations in adipose tissue gene expression and an increase in adipose tissue deposition [225]. However, the relative role of dietary n-3/n-6 PUFA, saturated fatty acids and trans fatty acids on early development of adipose tissue and their potential impact on childhood obesity has not been clearly investigated in humans and currently there is no robust evidence from animal studies to support the role of specific dietary fat types as early determinants of increased adiposity in the offspring. Therefore, the aim of the third chapter of this thesis was to investigate the effect of exposure to a maternal cafeteria diet on the proximate and fatty acid composition of the maternal milk and the relationship between maternal dietary intake, milk composition and offspring adiposity at weaning.

1.3.4 Cafeteria diet feeding - A robust model

Most of the investigations into the metabolic consequences of maternal obesity in rodents have been carried out through the administration of lard-based, high-fat diets [226], in which carbohydrate-derived calories are replaced with fat-derived calories, and comparing outcomes with those of rats fed a low fat or standard chow diet [227]. An alternative to these 'high-fat' diets is the use of a highly palatable cafeteria diet, consisting of a range of foods that are prevalent in the Western diet (e.g. cakes, pies, chips and chocolate) [227]. The cafeteria diet promotes voluntary hyperphagia which results in rapid weight gain, an increase in the fat pad mass and glucose and insulin intolerance [228-230]. Comparative studies between lard-based high-fat diets and cafeteria diets in diet-induced obesity have suggested that feeding rodents a cafeteria diet is the most clinically relevant approach for studying the impact of poor nutritional intake in humans, as these diets are more similar in composition to poor-quality

Western diets than diets that are exclusively high in fat or sugar [227]. Thus, the rapid onset of weight gain, increased fat deposition and other metabolic diseases observed in rodents fed on cafeteria diets reflect the situation seen in the human diet-induced obesity more closely than what occurs after feeding rats on lard based high-fat diets [227, 231].

1.4 THE ADIPOCYTE: AN IMPORTANT TARGET FOR DEVELOPMENTAL PROGRAMMING

1.4.1 Development and function of adipose tissue

It has been proposed that exposure to excess fetal nutrition during critical windows of development may result in the changes in the biology of adipocyte, which results in increased adiposity in later life [232]. It has been demonstrated in studies in sheep that an increase in maternal and hence fetal nutrition results in an increase in the expression of genes that regulate adipogenesis and lipogenesis and synthesis of adipokines in fetal adipose tissue, and these changes may be critical in the development of obesity in later life [233]. The importance of early postnatal overnutrition in the determination of later body composition has also been investigated in human studies, and these studies have demonstrated that nutrition during early postnatal development is imperative for later regulation of energy balance and fat deposition in human infants [145, 234]. However, the roles of these two respective periods in influencing gene expression/lipogenic capacity in the adipocyte are currently unclear.

1.4.1.1 *White and brown adipose tissue*

Adipose tissue depots begin as small clusters of lipid droplets that are surrounded by extensive stroma and a network of capillaries [235]. There are two main forms of adipose tissue, brown (BAT) and white (WAT). White adipocytes are spherical cells whose size largely depends on the amount of lipid stored in them, since lipid in the form of triglycerides (TGs) accounts for more

than 90% of the cell volume. Mitochondria present in white adipocytes are thin, elongated, and variable in amount. Brown adipocytes are typically polygonal with a variable diameter and, in contrast to white adipocytes, contain TGs in multiple small vacuoles [236, 237]. The most distinctive organelles of brown adipocytes are the mitochondria, which are usually numerous and are large, spherical and packed with laminar cristae. BAT also contains more capillaries than WAT to meet its greater oxygen demand. Compared to WAT, the nerve supply in BAT is also denser, and the brown colour of this tissue is attributable to its high mitochondrial density and high vascularisation [236, 237].

Both WAT and BAT are present in all mammals, and both forms of adipose tissue play an active role in the regulation of energy balance. The key function WAT is to store energy in the form of lipid, which can then be mobilised during times of negative energy balance to supply energy to other cells [238]. WAT also provides thermal insulation and has a role in inflammatory processes [239]. The physiology of WAT includes 3 potentially overlapping mechanisms; lipid metabolism, glucose metabolism and endocrine function. Lipid metabolism involves TG storage and fatty acid release, which is again controlled by 3 basic cellular functions; fatty acid intake, lipogenesis (fatty acid and TG synthesis) and lipolysis (TG hydrolysis). Glucose metabolism catabolises TGs in order to release glycerol and FAs into the circulation. Endocrine functions involve the secretion of adipokines, which include hormones, cytokines and other proteins with a wide range of biological functions [240]. BAT, on the other hand, has a completely different role, being involved primarily in non-shivering thermogenesis. BAT mediates this function through activation of a unique uncoupling protein (UCP-1) in brown adipose cells, which uncouples dissipation of the proton motive force from energy (adenosine triphosphate, ATP) production, instead releasing the energy in the form of heat [241, 242]. Thus, activation of BAT increases endogenous energy expenditure and thus counteracts weight gain and obesity [241, 242].

Adipose tissue mass changes dramatically in humans across the life course, and no other tissue has the same capacity to expand as does adipose tissue [243]. WAT is the predominant adipose tissue in adult humans and most adult mammals [244]. In contrast, BAT is the main form adipose tissue in humans during fetal development and in early postnatal life [245-248]. Although recent studies have confirmed the presence of BAT in adult humans, the amounts are much lower than those present in fetal and early neonatal life [244, 245, 249]. Rodents and hibernating mammals, on the other hand, possess considerable deposits of BAT throughout their adult life, since BAT in these animals plays an important role in assisting them in maintaining body temperature during prolonged cold exposure [250].

1.4.2 Adipose tissue development

In humans, the development of white adipocytes begins in the early embryonic stage [251] however, it is not until shortly after birth that most of the differentiation process occurs [252]. The development of brown adipocytes begins earlier at the 20^{th} week of pregnancy in humans and persists until a short time after birth, at which time BAT comprises 1% of body weight [241, 244, 253]. The adipose precursor cells originate from multipotent stem cells within the mesenchymal stroma which are then committed to the adipose cell lineage through differentiation [235]. Development of adipose cells and the mechanisms involved in adipocyte proliferation, differentiation and adipokine secretion has revealed the presence of two distinct cell lineages. One is pre-adipocytes, which are committed to the adipocyte lineage and the other are multipotent stem cells, which are able to commit to different lineages including adipose tissue, bone and muscle [235, 252]. Differentiation of pre-adipocytes to adipocytes is regulated by a series of transcription factors [254] and pre-adipocytes undergo a number of stages of differentiation before they become fully mature adipose cells [235, 255-257].

1.4.3 Regulation of adipogenesis

Adipogenesis is a controlled multi-step process requiring the sequential activation of several hormones, growth factors and groups of transcription factors. Adipocyte differentiation is characterised by orderly changes in the expression of particular genes which determine the specific adipocyte phenotype of the cells [258-260].

1.4.3.1 *Determinants of adipose cell maturation*

Hormones and growth factors that are involved in the differentiation of adipocytes act via specific receptors, which mediate external growth and differentiation signals via a number of intracellular events. Both autocrine and paracrine mediators have a major role in the stages of adipocyte maturation [261, 262] and the most important autocrine factors involved in adipocyte differentiation are insulin, insulin-like growth factor 1 (IGF-1), glucocorticoids, thyroid hormone and cyclic adenosine monophosphate (cAMP) [263, 264]. Previous studies have clearly demonstrated the requirement for both IGF-1 and insulin in adipocyte differentiation [258, 259, 265]. The IGF-1 receptor is expressed at high levels on the surface of pre-adipocytes, and maturation of pre-adipocytes *in vitro* can be induced by the addition of IGF-1 [259]. The effect of insulin on differentiation occurs via cross-activation of the IGF-1 receptor and a number of distinct downstream signal transduction pathways, activated by both IGF-1 and insulin, mediate the adipogenic effects of these hormones [256, 266]. The expression of the insulin receptor increases during adipocyte maturation and the adipose cells become progressively more reactive to the actions of insulin at physiological concentrations [256, 266]. Pre-adipocytes also express the glucocorticoid receptor and glucocorticoids have been used widely to induce the maturation/differentiation of cultured pre-adipocyte cell lines and primary pre-adipocytes [267]. Growth hormones (GH) and other growth factors, including, epidermal growth factor (EGF), transforming growth factor-alpha (TGFα) and transforming growth factor-beta (TGFβ), have also been shown to be involved in the regulation of adipocyte differentiation and previous studies

have reported that depleting culture medium of GH and other growth factors results in arrested differentiation of pre-adipose cells [261, 262, 268, 269].

Adipose cell differentiation and adipose cell function is also regulated by paracrine factors, notably acylation-stimulating protein (ASP), angiotensinogen and prostaglandins PGE_2 and PGI_2, which promote differentiation, and TGFα, which inhibits differentiation [262, 270].

1.4.3.2 *Transcriptional regulation of adipocyte differentiation*

A series of transcriptional processes are involved in terminal adipocyte differentiation and maturation and two important transcription factors, CCAAT/enhancer-binding protein-beta (C/EBPβ) and CCAAT/enhancer-binding protein-gamma (C/EBPγ) have been shown to activate adipocyte-specific genes, and are also involved in the growth arrest that is required for adipocyte differentiation/maturation [262]. These transcription factors are expressed in proliferating pre-adipocytes but are absent following the growth arrest stage and appear to participate in the switch from proliferation to differentiation during adipocyte maturation [262]. They also appear to act cooperatively in adipose differentiation, by activating the expression of one another and also regulating the expressions of other adipocyte specific genes [271]. Most specific to adipogenic differentiation is the peroxisome proliferator-activated receptor gamma (PPAR-γ), and is induced before transcriptional activation of most other adipocyte genes [256]. The ectopic expression of C/EBPβ in NIH-3T3 cells upregulates the expression of PPAR-γ *in vitro* [272] and ectopic expression of CCAAT/enhancer-binding protein-alpha (C/EBPα) in fibroblasts can induce adipogenesis only in the presence of PPAR-γ [273]. It has also been reported that the ectopic expression of PPAR-γ can induce adipogenesis in mouse embryonic fibroblasts deficient in C/EBPα, but C/EBPα cannot rescue adipogenesis when PPAR-γ is not expressed [274]. PPAR-γ is highly expressed in adipose cells early in development and the action of PPAR-γ is mediated through the main PPAR-γ isoforms: PPAR-γ1 and PPAR-γ2 [256]. The PPARs

promote gene expression by forming heterodimeric complexes with the retinoid-acid receptor (RXR), which then migrate to the nucleus and interact directly with the specific DNA regions. The final stages of adipocyte maturation involve the activation of genes which encode factors significant for metabolic and physiological function of the mature adipocyte; including those genes encoding the adipokines e.g. leptin and adiponectin, and those involved in fatty acid synthesis [256] (Figure 1.5).

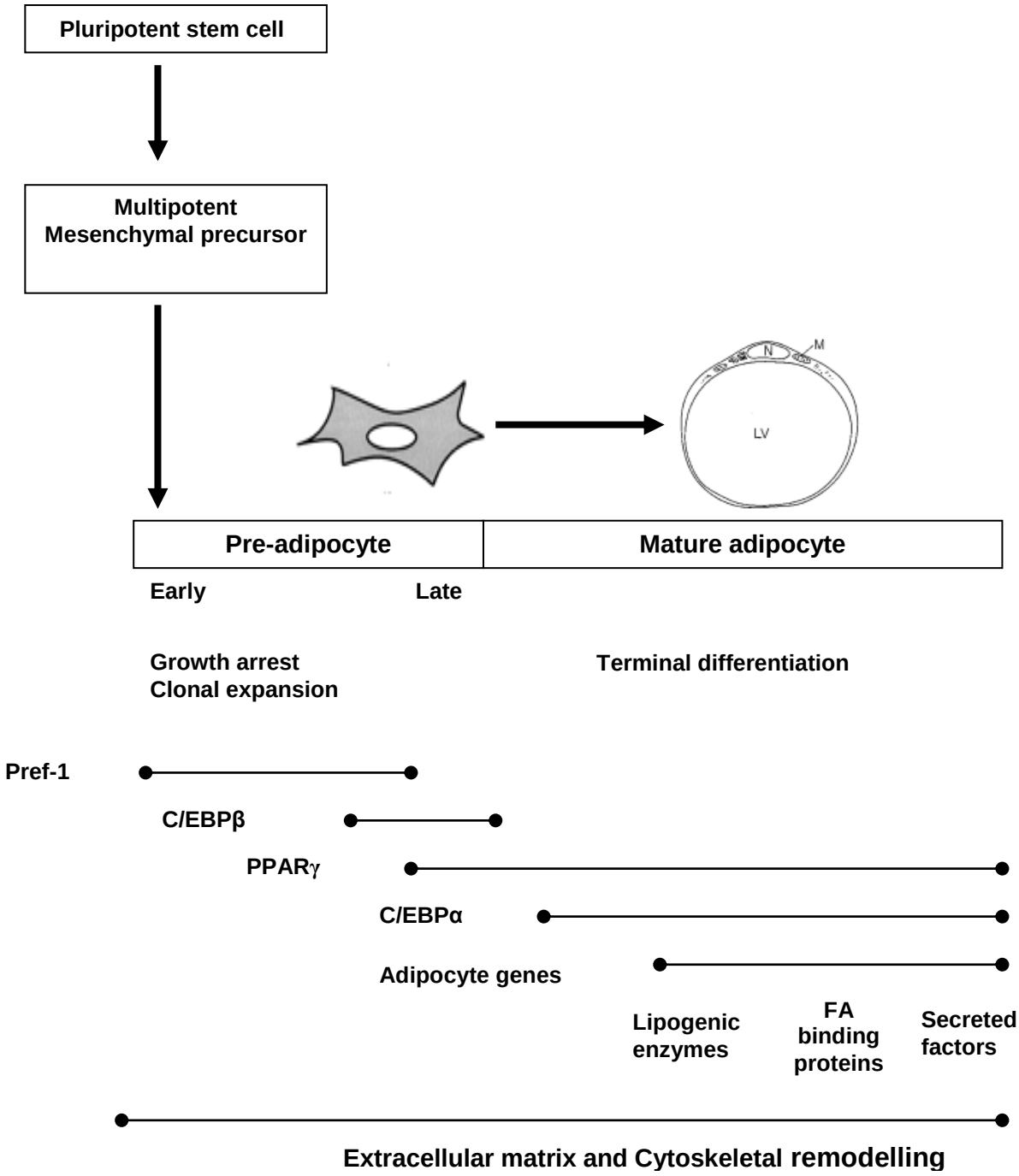


Figure 1.5 Overview of stages in adipocyte differentiation**.**

PPAR-γ : peroxisome proliferator-activated receptor-γ; **C/EBP**: CCAAT/enhancer binding protein; **pref-1**: pre-adipocyte factor, **FA** : fatty acid

Adapted from Gregorie et.al, 1998. [256]

1.4.4 Regulation of lipogenesis and lipolysis

The balance between lipogenesis (lipid synthesis) and lipolysis (fatty acid oxidation) determines the amount of fat which is stored in adipose cells [275]. When the rate of lipogenesis exceeds the rate of lipolysis, body fat mass will increase and thus excess storage of body fat will occur. In other words, it is ultimately due to the chronic positive energy balance as a result of either excessive energy intake, a reduced total energy expenditure or a combination of both [276]. Lipogenesis takes place in both liver and adipose tissue and encompasses the processes of fatty acid synthesis and subsequently the synthesis of TGs which are the main form of fat storage in adipose tissue [275, 277] (Figure 1.6). A number of metabolic, endocrine and hormonal factors play a key role in the regulation of lipogenesis and lipolysis and the levels of these factors are mostly dependent on current nutritional status [275].

1.4.4.1 *Transcriptional regulation of lipogenesis*

Lipogenesis is under the coordinated control of several transcription factors which have been identified as critical regulators that mediate the effect of various hormones and nutrients on the expression of different lipogenic genes. SREBPs, C/EBPα, the nuclear hormone receptors–liver X receptors (LXR), PPAR-γ and the estrogen related receptor alpha (ERRα) each play a significant role in the regulation of lipogenesis, with sterol regulatory element binding protein 1-c (SREBP-1c) and PPAR-γ considered the most important [275]. Previous studies have suggested that SREBPs mediate the effects of various nutrients and hormones on the expression of a number of lipogenic genes [278-280]. SREBPs regulate the expression of genes associated with cholesterol and fatty acid metabolism and they can be separated into three sub-types: SREBP-2, SREBP-1a and SREBP-1c (also known as adipocyte determination and differentiation-1, ADD-1), of which, SREBP-1c is considered to be the most physiologically relevant for adipose tissue lipogenesis.

Previous studies have reported that SREBP-2 stimulates the expression of genes involved in cholesterol metabolism and SREBP-1a activates genes associated with lipogenesis in liver [281]. In adipocytes, SREBP-1c most likely works in co-operation with the adipogenic transcription factor PPAR-γ, which is a direct target of SREBP-1c in this tissue [282]. SREBP-1c is able to upregulate PPAR-γ expression and is also able to induce PPAR-γ activity by the production of an endogenous ligand which further leads to the stimulation of adipogensis and lipogenesis [283]. Other important lipogenic genes such as acetyl-CoA Carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), glycerol-3-phosphate acyltransferase (GPAT) and low density lipoprotein receptor (LDL-R) have also been identified as direct targets of SREBP-1c in mature 3T3-l1 adipocytes [284].

PPARs play a critical role in the regulation of lipogenesis and the expression of this protein is further activated by fatty acids [285], insulin [286] and SREBP-1c [282]. Once activated, PPAR-γ upregulates the expression and activity of genes involved in increasing storage of lipids in adipose cells including, fatty acid binding protein, lipoprotein lipase (LPL), fatty acid transport protein (FATP), acyl-CoA synthetase, phosphoenolpyruvate carboxykinase (PEPCK) and the fasting–induced adipose factor FIAF/ PPAR-γ angiopoietin related peptide (PGAR) [287, 288]. PPAR-γ ligands also reduce the expression of leptin and increase the expression of the insulin-sensitising hormone, adiponectin, in adipose cells [289].

1.4.4.2 *Enzymes involved in lipogenesis*

Several enzymes are involved in the *de novo* synthesis of fatty acids, the most important being ATP-citrate lyase, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), malic enzyme (ME), glycerol 3-phosphate dehydrogenase (G3PDH) and glucose-6-phosphate dehydrogenase (G6PD). The substrate for TG synthesis is the newly synthesised fatty acids or free fatty acids (FFA) taken up into adipose cells from the circulation [290] and the activity of these enzymes

is co-ordinately regulated such that TG synthesis is high during times of positive energy balance and low during negative energy balance, when fatty acids are being utilised as an energy source [275, 277].

Of the many enzymes involved in the *de novo* synthesis of fatty acids, FAS, plays a central role in *de novo* lipogenesis in mammals [291]. FAS is not a single enzyme but a whole enzymatic system composed of two identical 272 kDa multi-functional polypeptides, in which substrates are handed from one functional domain to the next [292-294]. FAS catalyses seven different activities needed for the conversion of acetyl-CoA and malonyl-CoA to palmitate [295, 296]. FAS expression is acutely sensitive to nutritional and hormonal status in lipogenic tissues, liver and adipose tissue [291, 297, 298] and the amount of FAS protein present in both liver and adipose tissue is largely regulated by the rate of FAS synthesis in these organs [299-301]. FAS mRNA expression decreases during fasting, and increases rapidly upon re-feeding, and these effects are thought to be mediated by glucagon and insulin respectively [298]. Low levels of FAS expression are present in animals with T2DM, and this can be reversed by the administration of insulin [298, 302]. Depressed FAS activity is also reported in starved animals, which increases rapidly upon re-feeding a fat-free diet [299, 300, 303].

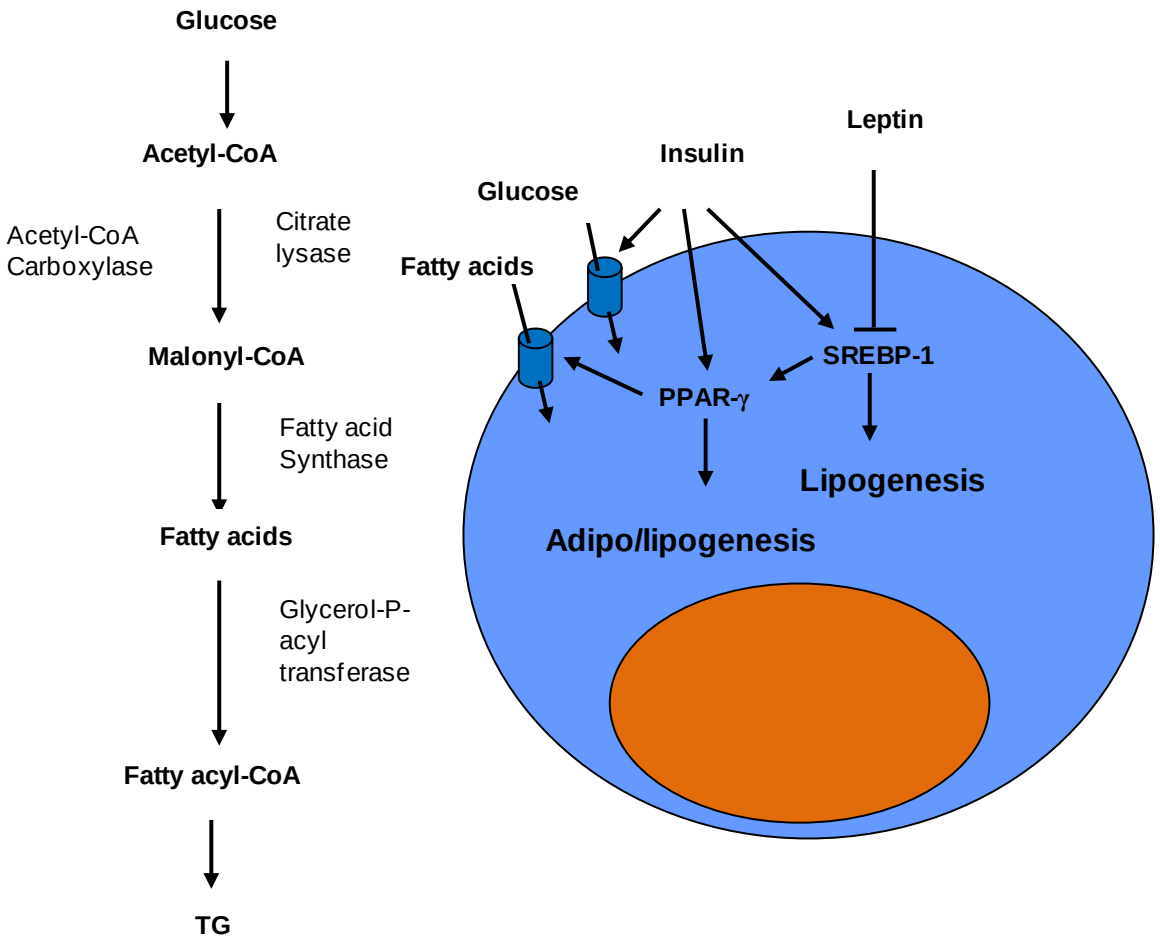
1.4.4.3 *Hormonal regulation of lipogenesis*

Glucose and insulin are the two most important physiological regulators of lipogenesis [275]. Insulin increases the cellular uptake of glucose in adipose cells by promoting the recruitment of glucose transporters to the plasma membrane and also activates the key lipogenic and glycolytic enzymes (FAS and ACC) via covalent modification [265, 304, 305].

Glucose acts directly to increase the expression of principal lipogenic genes and also acts as a substrate for the *de novo* synthesis of fatty acids; acetyl-coA, the metabolic product of glucose metabolism, is the precursor for fatty acid

synthesis in adipose tissue [275, 306]. Both glucose and insulin increase the expression and activity of SREBP-1c, which then activates the expression of a number of lipogenic enzymes [275].

GH also has an important role in regulating lipogenesis, and acts to reduce lipogenesis in adipose tissue which results in significant loss of fat and parallel gain of muscle mass [307]. Another hormone that influences lipogenesis is the adipocyte derived hormone, leptin. Leptin stimulates fatty acid oxidation/fat mobilisation and inhibits lipogenesis by down-regulating the expression of lipogenic genes [308, 309] and thereby stimulates the release of glycerol from adipocytes [310]. Recent studies have also reported that leptin inhibits the activity of SREBP-1c, suggesting the involvement of this transcription factor as a mediator of the inhibitory effect of leptin on lipogenic gene expression [311, 312].



Adapted from Kersten S, 2001. [275]

Figure 1.6 Regulation of lipogenesis in adipocytes. The effects of nutrients and hormones on the expression of lipogenic genes are mostly mediated by PPAR-γ. Lipogenesis entails a number of discrete steps, shown in the left, which are controlled via allosteric interactions, by covalent modification and via changes in gene expression.

1.4.4.4 *Lipolysis*

Lipolysis involves the hydrolysis of triacylglycerol (stored in the form of TGs) into FFA and glycerol, which can then be released from adipose tissue and used by other organs as energy substrates [313]. Under normal physiological conditions, lipogenesis and lipolysis are in balance, and there is no net increase or decrease in adipose tissue mass. Lipolysis increases in response to negative energy balance, to supply the energy needs of other tissues. During lipolysis, adipose triglyceride lipase (ATGL) hydrolyses the triglyceride stores in the lipid droplets to release diacylglycerol and fatty acids [314]. The diacylglycerols are then hydrolysed by hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) to produce free fatty acids and glycerol [315]. HSL is constitutively expressed in adipose cells and is activated via protein kinase A-dependent phosphorylation [316]. HSL activation is regulated by several circulating factors, including catecholamines (adrenaline and noradrenaline), GH, glucagon and thyroid hormone, which stimulate lipolysis, and insulin, which inhibits it [275]. The rate of lipolysis in WAT is also increased by leptin [309, 316]. The sensitivity of adipocytes to cathecholamine-mediated lipolysis is also influenced by their anatomical location such that, compared to subcutaneous adipose tissue, the visceral adipose tissue is more sensitive to lipolytic signals [317, 318].

1.4.5 Adipose tissue as an endocrine organ

Historically, the physiological role of WAT was seen as primarily one of energy storage, as it provided a long-term fuel reserve which could be liberated during times of negative energy balance. However in recent years, it has become clear that WAT also has an important endocrine function. WAT has been shown to secrete a wide array of hormones and cytokines (known as adipokines) and is recognised as having a fundamental role in the control of whole-body energy metabolism. Although many of the factors secreted from adipose tissue regulate adipocyte metabolism in an autocrine/paracrine manner, some other factors are released into the bloodstream and influence the function of other organs and tissues [319, 320]. The number of adipokines to be identified has expanded

rapidly during the recent years, and include: leptin, adiponectin, resistin, serpin, lipocalin-2, Pai-1, RBP4, ZN α-2 glycoprotein, vaspin, visfatin, omentin, apelin and chemerin, all of which have systemic effects [321]. Of these many adipokines, leptin and adiponectin have attracted particular attention due to their role in the control of energy balance, insulin sensitivity and appetite.

1.4.5.1 *Leptin*

Leptin is a 16kDA polypeptide hormone secreted principally by the adipocytes, and was first characterised in 1994 by Friedman and colleagues [322]. Leptin is secreted into the circulation in proportion to total body fat mass, and therefore acts as a circulating signal of body fat stores [323-325]. Leptin concentrations in the circulations of adult humans, rodents and sheep are positively correlated with measures of adiposity, including percentage body fat mass and fat cell size [326-328]. Leptin is considered as a metabolic signal for energy sufficiency as it regulates energy metabolism increasing energy expenditure and decreasing energy intake. The important role of this circulating factor in the regulation of whole energy balance in adult rodents was first demonstrated by Coleman, who connected the circulations of leptin deficient mice (*ob/ob*) with the circulations of mice who were deficient in leptin receptor(*db/db*) [329]. Following this surgery, leptin deficient mice lost body weight and fat mass rapidly while those mice that were deficient in the leptin receptor were unaffected. Coleman concluded from these studies that *ob/ob* mice were deficient in a circulating factor whose receptor was absent in *db/db* mice [329], and the ob (leptin) gene product was subsequently sequenced and purified [322]. The role of leptin in the regulation of fat mass and food intake has since been confirmed in studies in adult rodents, in which central or peripheral infusion of leptin has been demonstrated to inhibit food intake and weight gain [330], decrease the mass of WAT [331, 332], promote lipolysis and reduce lipogenesis in white adipocytes and increase thermogenesis in brown adipocytes [333]. The overall rate of insulin-stimulated glucose utilisation and the metabolic rate in the adipose tissue is also increased by leptin infusion such that, in the adipose cells, nutrients are utilised for cellular activities rather than being stored [330, 333, 334]. Previous studies have also

established that leptin infusion increases the rate of lipolysis independently of leptin-induced changes in appetite and food intake [310] indicating that it has a direct effect on adipocyte metabolism.

Paradoxically, leptin concentrations are significantly higher in obese individuals than in lean individuals, but fail to suppress food intake or prevent further increases in fat mass effectively. It has now been demonstrated in both humans and animal models that exposure to elevated leptin levels rapidly leads to the development of a resistance to its actions [335, 336]. The resulting leptin resistance has been suggested to be an important factor which contributes to the development of human obesity and resistance to weight loss [336]. Importantly for this thesis, early programming of leptin resistance has also been implicated in the programming of the obese phenotype by nutritional exposures *in utero*.

Previous studies in humans [337, 338] and sheep [339] have reported that leptin has a potential role as a signal of fat stores before birth. In both humans [340] and sheep [341, 342], changes in fetal plasma leptin concentrations occur in parallel with changes in intrauterine fat deposition and there is a positive correlation between leptin concentrations in the umbilical cord blood at delivery and measures of neonatal adiposity including BMI, ponderal index and subscapular skinfold thickness in human infants [337, 338]. In early postnatal life, however, a distinct ontogenic profile of circulating leptin concentrations has been reported in many, but not all, studies in rodents, sheep and humans, such that plasma leptin concentrations are higher in the neonate than in the adult immediately after birth, and increase to a peak within the first few days after birth, before decreasing to adult levels by the end of the first month of life [343-347]. A nutritional influence on the ontogenic profile of leptin in the neonatal circulation has also been reported in lambs [347].

1.4.5.2 *Adiponectin*

Adiponectin is a secreted protein of 244-amino acids, released exclusively by adipocytes [348]. Adiponectin is a protein hormone that modulates a number of

metabolic processes, including glucose metabolism, insulin signalling and fatty acid oxidation [349]. Structurally adiponectin has a striking similarity to tumour necrosis factor-α (TNF-α) despite its unrelated protein sequences [350]. This protein also has sequence homology to the complement factor C1q [351, 352]. Adiponectin is secreted into the blood stream with concentrations between 2 and 20 $\mu g/mL^{-1}$ where it accounts for up to 0.05% of total serum protein and compared to many other hormones adiponectin is very abundant in the plasma [348, 353]. Plasma concentrations are also sexually dimorphic, with females having higher levels than males [353, 354]. Adiponectin was first characterised in 1995 in differentiating 3T3-L1 adipocytes [348] and in later years it was characterised as the mRNA transcript most highly expressed in adipocytes and pre-adipocytes differentiating into adipocytes [355-357].

Adiponectin has a number of biological functions which include insulin sensitising [358], anti-atherogenic [359], anti-inflammatory [360], anti-angiogenic and anti-tumour effects [361]. Unlike leptin, which is elevated in obesity, plasma adiponectin levels are lower in obese individuals than in lean individuals and it has been reported that there is a strong negative correlation between adiponectin levels in the plasma and BMI in both humans and animals [353, 354, 362]. The negative correlation between visceral adiposity and circulating adiponectin is even stronger than for total fat mass, and visceral adiposity has been shown to be an independent negative predictor of adiponectin levels in the circulation [363-365]. The reduced level of adiponectin in obese individuals is thought to be one of the factors contributing to the reduced insulin sensitivity and increased risk of T2DM in obese individuals [354, 358, 366-368]. Whilst the levels of the adiponectin are reported to be inversely correlated with body fat percentage in adults, this does not appear to be the case in infants and young children. Adiponectin in infants and children is reported to be higher than in adults and to be positively related to neonatal body weight/fat mass [369, 370], suggesting that adiponectin may play an important role in promoting insulin sensitivity and deposition of fat tissue in infancy and early childhood [369, 370].

1.4.6 Adipose cell development in the offspring: The importance of the prenatal and early postnatal periods in humans, sheep and rodents

In humans, the process of white adipocyte development from stem cell precursors begins before birth, and continues into the first year of life [235, 257]. In addition, there is a second period of accelerated fat differentiation/deposition at around 6 years of age (termed the adiposity rebound), the timing of which is considered to be important for determining the later risk of adult obesity [371]. Previous studies have also reported that the capacity of pre-adipocytes to proliferate and differentiate during adult life is much lower than before birth, in infancy and in early childhood and that most, if not all, adipose tissue development is completed in prenatal and early postnatal life [235, 257]. This therefore implies that the fetal and early postnatal periods are critical windows in the development of adipose depots.

In humans, WAT is well developed in both the visceral and subcutaneous depots by birth. In rodents, while expression of adipocyte genes is present in pre-adipocytes before birth, the characteristic morphology of adipocytes develops only in the first 24 hours after birth. WAT cannot be detected macroscopically during fetal life or at birth in the rodent, first appearing in the perigonadal and subcutaneous depots, and only later in the omental depot after the pups are born [372]. The fat content of newborn rat varies between 1 and 2% of the body weight and most of this fat is a component of body tissues [373]. BAT, in contrast, emerges earlier than WAT during fetal development and reaches its maximal size relative to body weight at birth (mainly in the interscapular region), when non-shivering thermogenesis is required. Later, it involutes with age, in both humans and rodents. Although dissectible fat depots are not present in rats at birth, rat adipocytes are formed earlier in development, with the expression of adipose-specific genes detected late in gestation and accumulation of triacylglycerol in brown adipocytes also occurring *in utero* [374].

The process of adipocyte development is highly sensitive to the fetal and early postnatal nutritional environment. Studies in sheep, which have a similar profile of adipose cell development to humans, have reported that exposure to increased glucose concentrations in late fetal life increases the expression of genes within adipose cells which promote lipid storage and formation of new adipocytes [375]. Importantly, this change in the expression of adipogenic/lipogenic genes in late fetal life was associated with an increased adipose tissue mass by the end of the first month of life, largely owing to an increase in adipocyte cell size in lambs who had been exposed to the same degree of maternal overnutrition prenatally [76]. Currently, there is evidence from studies in both humans and sheep that relatively minor perturbations in fetal adipose tissue growth and endocrine sensitivity may have important long-term effects on adipose tissue mass [235, 257, 376]. Furthermore, the magnitude of these changes in the adipose tissue is mainly determined by the maternal and fetal nutritional environment [235, 257, 376]. While programming of adipocyte function and gene expression is considered to play an important role in early life origins of obesity, the relative role of the prenatal and early postnatal environments in these effects is currently unclear.

1.5 SEX DIFFERENCES

According to 2014 World Health Organization fact sheet, obesity prevalence is different between male and females, with females having higher rate of obesity than males [4]. Human observational studies reported that females have higher responsiveness to stimuli that increase the drive to eat [377] and are also more resistant to weight loss [378, 379]. Sexual dimorphism has been described not only in body weight control but also in body composition, fat distribution and fuel metabolism [380, 381]. Previous studies have reported that females have lower resting energy expenditure than males [382] and it is more likely to continue to reduce with age, making females more prone to weight gain and obesity [383]. Sex differences are also present in relation to fat distribution, with males tending to deposit more visceral fat and females tending to deposit more subcutaneous fat [384]. It has been reported that the difference in body morphology, in

particular, the differences in fat distribution between the sexes, underlies the sex-related differences in the prevalence of chronic diseases [378]. Thus, females are more likely than males to develop obesity in response to intake of poor quality (high-fat, high-sugar) diets [378, 379], while males are more likely to develop obesity related metabolic disorders, including cardiovascular disease and T2DM [385].

Despite these significant differences between males and females in the susceptibility to obesity and metabolic diseases, the majority of studies in the obesity field, particularly experimental studies aimed at addressing the underlying mechanisms, have been conducted only in males. The justification for this has been that it avoids the hormonal fluctuations which occur as part of estrous cycle in females, which could potentially confound the result [386, 387]. As a result, however, there is currently limited knowledge as to whether the mechanisms which underlie the early programming of obesity are the same between males and females, and further studies are required.

1.5.1 Sex differences in the programming of obesity in response to maternal under/overnutrition during the prenatal and postnatal period

Although it is clear from previous studies that sexual dimorphism exists in adult energy homeostasis in response to high-fat diet, it was not clear until relatively recently that the effects of exposure to an early nutritional insult on long-term risk of obesity and poor metabolic health also differs between males and females. Previous animal studies of maternal undernutrition have reported that, in comparison to females, males are more prone to develop hypertension and impaired glucose homeostasis in later life [388-390]. The Dutch Hunger Winter Famine study also reported a higher incidence of obesity in men whose mothers were exposed to the famine during the first 2 trimesters of gestation compared to women [53]. Jones and Friedman observed that male offspring of rat dams who underwent 50% caloric restriction during the first 2 weeks of pregnancy became obese after 5 weeks of age, whereas female offspring did not [391]. However, not all studies have produced similar results. A study by Anguita and

colleagues [392], for example, reported that a intrauterine malnutrition in dams during the first 2 weeks of gestation led to increased fat accumulation throughout the postnatal period in the males, whereas the female offspring showed increased accumulation of fat only in adulthood. This was observed in spite of the fact that both sexes exhibited normal levels of food intake [392].

There have been even fewer experimental studies of maternal overnutrition which have included both male and female offspring. Bayol and colleagues directly compared the outcomes of male and female offspring, and reported that the impact of exposure to a maternal cafeteria diet *in utero* and during the suckling period on body weight, fat deposition and expression of lipogenic genes was greater in female compared to male offspring, although both sexes were heavier and fatter than their control counterparts [117]. Another study reported that feeding dams a lard-based diet from before pregnancy until the end of lactation resulted in the development of endothelial dysfunction in both male and female offspring, but that only the female offspring were hypertensive [102]. Samuelsson and colleagues also demonstrated that only the male offspring of mothers exposed to overnutrition during pregnancy and lactation developed impaired glucose homeostasis during adulthood [17]. There were no sex differences, however, in the effects of the maternal diets on the development of hypertension in the adult offspring in this same study. Another study reported that female offspring of obese mothers who were calorie restricted before pregnancy and during pregnancy and lactation were resistant to diet-induced obesity during adulthood but that males were more susceptible to diet-induced obesity [393]. Interestingly, another study reported a protective effect of maternal high-fat feeding on later obesity risk in male offspring, such that male offspring gained less weight and exhibited metabolic parameters similar to that of controls at 10 weeks of age, even when they were fed a high-fat diet after weaning [101]. This study also reported, however, that both male and female offspring of obese dams were less sensitive to leptin compared to control, when all offspring were weaned to a control diet, suggesting that maternal overnutrition during pregnancy and lactation programs leptin resistance in the offspring independent of offspring sex [101].

While not all studies have produced consistent results, there is nevertheless evidence that sexual dimorphism exists in the susceptibility to diet-induced obesity in response to maternal overnutrition/obesity. However, the separate contributions of maternal nutritional environment during the prenatal and postnatal period in regards to the sex differences in the susceptibility to diet-induced obesity have yet to be determined. It is also unclear whether this sexual dimorphism is due to a sex specific regulation of genes related to metabolism in response to maternal overnutrition during development or it is linked to the difference in the susceptibility to metabolic disorders between males and females.

1.6 SUMMARY

A large number of studies have now modelled maternal nutritional-excess by means of a number of different approaches, which all resulted in an increase in the incidence of adult obesity although the timing and the magnitude of the phenotype vary according to the nutritional insult. More recently, the attention of the Developmental Origins of Health and Disease (DOHaD) field has increasingly turned towards evaluating the impact of maternal obesity and nutritional-excess, and these studies have provided strong evidence that this has important negative impacts on the long-term health of the offspring [93, 117, 394-396]. Most of the experimental nutritional studies to date support the notion that there is a U-shaped curve present in the relationship between the level of maternal nutrition, offspring birth weight and risk of metabolic diseases in postnatal life with an increasing incidence of adult obesity in individuals who were exposed to either an inappropriately low or inappropriately high plane of maternal nutrition during the prenatal period [17, 89, 93, 104, 397]. Exposure to increased maternal nutrition during the perinatal period has also been reported to lead to programmed alterations in genes important for adipocyte differentiation and function in the adult offspring [17].

More recently, evidence has emerged that not just the prenatal, but also the early postnatal environment is important for determining the future risk of obesity

and that the nutritional environment experienced by individuals during the early postnatal period also has a significant effect on the long-term development of organ systems which predisposes the offspring to obesity and other metabolic diseases [398]. It was reported by Kozak and colleagues [398] that the gene expression phenotypes established in individual mice during development are re-expressed in adult mice, suggesting that the nutritional environment during early postnatal development controls the capacity of the adipocyte to accumulate fat by modulating the expression of a subset of specific regulatory genes, and that this capacity for structural remodelling is recapitulated in the adult mouse.

Since the fetal nutrient supply and breast-milk composition are closely related to maternal dietary intake, significant changes in the composition of the maternal diet could potentially result in a substantial increase in the consumption of saturated fatty acids, trans fatty acids and n-6 PUFA relative to n-3 PUFA, reflected in the breast-milk, and may be important in controlling fat deposition in the growing pup. The programming of adipose gene expression may also be a mechanism through which the exposure to overnutrition during development leads to increased adiposity in adult life. Understanding the mechanisms behind this programming of increased fat mass in the offspring has centred on the effect of maternal cafeteria feeding on breast-milk composition, offspring adiposity and, given the importance of adipose tissue in the programming of obesity in adults, on the expression of key adipogenic and lipogenic genes. Thus the aims of the current thesis are;

- To investigate the relative contribution of exposure to a maternal cafeteria diet during the prenatal and suckling periods to body weight, fat mass and glucose tolerance in the offspring at weaning and in early adolescence.
- To determine the effect of maternal cafeteria feeding on the composition of breast-milk and on the fatty acid status of the offspring at weaning and in early adolescence, and how these changes relate to early fat deposition.

- To investigate the relative importance of exposure to a maternal cafeteria diet before birth and during suckling period on the expression of adipocyte regulatory genes in the offspring at weaning, in adolescence and in young adulthood.
- To determine the relative contribution of exposure to a maternal cafeteria diet before birth and during the suckling period to the susceptibility of the offspring to diet-induced obesity in young adulthood.
- To compare these outcomes between male and female offspring.

1.7 EXPERIMENTAL HYPOTHESES

Whilst there was considerable evidence that exposure to an increased nutrient supply during different periods of development, specifically, before birth and in the early postnatal period has differential effects on the metabolic health of the offspring, the majority of studies to date had investigated the consequences of maternal obesity/maternal high-fat diets during both the prenatal and postnatal periods [15-17]. Moreover, studies which investigated the separate contributions of prenatal and early postnatal exposure had produced conflicting results with some reporting that exposure to a high-fat diet during the early postnatal period was more important for metabolic programming than exposure before birth [106], while others reported the opposite [116]. Thus, the relative contribution of the prenatal and postnatal nutritional environment to the programming of obesity remained unclear. **In Chapter 2, I used a cross-fostering paradigm, in which the offspring born to Cafeteria diet fed dams are fostered at birth to Control dams and vice versa, to investigate the relative effect of prenatal vs postnatal exposure to a high-fat/high-sugar cafeteria diet on offspring adiposity and other metabolic outcomes. I hypothesised that offspring suckled by CAF mothers would have higher body fat mass than offspring suckled by Control mothers at weaning, independent of they were born to CAF or Control dam. I further hypothesised that postnatal nutritional interventions would partially, but not completely, reverse the adverse effects of early exposure to a high-fat/high-sugar cafeteria diets before birth. I therefore investigated the relative contribution of exposure to a**

maternal cafeteria diet during the prenatal and suckling periods on body weight, fat mass and glucose tolerance in the male and female offspring at weaning and in early adolescence.

Whilst it had become increasingly clear that exposure to a maternal high-fat/high-sugar diet during the entire perinatal period was associated with an increased risk of obesity and metabolic disease in the offspring in postnatal life [399, 400], a small number of studies had also suggested that the exposure to these obesogenic diets during the suckling period alone would produce detrimental effects to offspring metabolic health which were similar compared to those exposed during the entire perinatal period [106, 107]. Since maternal milk is the dominant source of nutrition for the offspring during the suckling period, these findings suggested that alterations to milk composition as a result of inappropriate maternal nutrition may make an important contribution to these adverse effects in the offspring. However, it was largely unknown whether the composition of maternal obesogenic diets, which typically contains a higher proportion of total fat, sugar, saturated and n-6 PUFA and a lower n-3 LCPUFA and protein content [159, 189, 195, 401-404], could affect the macronutrient and fatty acid composition of the breast-milk, and whether these changes are related to key metabolic health outcomes, in particular fat mass, in the offspring. Previous studies had shown that the proximate and fatty acid composition of breast-milk is affected markedly by the type of diet available during lactation [159, 189] and that, of all the components of the maternal diet, the amount of fat and the content of individual fatty acid classes are most closely reflected in their levels in breast-milk [190, 191, 402, 405-407]. However, it was unknown how the fatty acid composition of these maternal obesogenic diets affected the fatty acid composition of the milk and the fatty acid status of the offspring. **In Chapter 3, I hypothesised that maternal consumption of a cafeteria diet during lactation would significantly alter the proximate and fatty acid composition of maternal milk and that these changes would be related to fat mass of the offspring at weaning. I further hypothesised that exposure to a cafeteria diet during the suckling period alone would change the fatty acid status of the offspring suckled by cafeteria-fed dams, irrespective of**

whether or not they were also exposed to the cafeteria diet *in utero* and that these changes would be related to the maternal dietary fatty acid intake during lactation and fatty acid composition of maternal milk. In Chapter 3 of this thesis, I therefore investigated the relative contribution of exposure to a maternal cafeteria diet during the prenatal and suckling periods on fatty acid status in the offspring at weaning and in early adolescence and the relationship of maternal dietary fatty acid intake during lactation and fatty acid composition of maternal milk to fat deposition and fatty acid status in the offspring.

There was considerable evidence from studies in both humans and animal models that exposure to increased maternal nutrition during critical windows of development played a role in the development of obesity, and that this may be the result of alterations in the biology of adipocyte [91, 117, 235, 257, 376, 400]. Previous studies had also reported that the process of adipocyte development is highly sensitive to the fetal and early postnatal nutritional environment [375] and that the fetal and early postnatal periods are critical windows in the development of adipose depots [235, 257]. However, there were no studies that had compared the relative contribution of exposure to a high-fat/high-sugar cafeteria diet before birth and during the early postnatal period on the expression of adipocyte regulatory genes in the offspring. It was also not known whether such changes may contribute to the later development of an obese phenotype if the offspring were weaned to a control diet. **In Chapter 4, I hypothesised that exposure to a cafeteria diet during the suckling period would alter the expression of key adipogenic, lipogenic and adipokine genes within the adipose tissue to increase the subsequent lipogenic capacity of fat depots to a greater extent than exposure before birth. I further hypothesised that providing a nutritionally balanced diet post-weaning would reverse these alterations in adipogenic, lipogenic and adipokine gene expressions in the adipose tissue**. **I therefore investigated the relative contribution of exposure to a maternal cafeteria diet during the prenatal and suckling periods on the expression of key adipogenic and lipogenic genes in adipose tissue in the offspring at weaning and in early adolescence.**

A large number of studies had confirmed that the susceptibility to develop obesity in later life is increased in response to exposure to maternal overnutrition, in particular excess maternal intakes of fat and/or sugar, experienced during the perinatal period [93, 117, 394-396]. Whilst there had been growing interest in determining the separate contributions of exposure to high-fat/high-sugar diets *in utero* and in the early postnatal period on the short and longer term metabolic health outcomes of the offspring [102, 103, 107, 108], in the vast majority of existing studies, the offspring had been exposed to increased maternal nutrition/maternal obesity during both the fetal and suckling periods and consequently the separate contributions of prenatal and postnatal periods to the programming of obesity remained unclear. **In Chapter 5, I hypothesised that exposure to a cafeteria diet during the suckling period would result in higher body fat mass in the offspring in young adulthood, independent of their nutritional exposure before birth. I further hypothesised that exposure to an increased nutrient supply during the suckling period would alter the expression of key adipogenic, lipogenic and adipokine genes within the adipose tissue to increase the subsequent lipogenic capacity of fat depots in young adulthood than exposure before birth. I therefore investigated the relative contribution of exposure to a maternal cafeteria diet during the prenatal and suckling periods on body weight, body fat mass and expression of key adipogenic and lipogenic genes in adipose tissue in the offspring in young adulthood.**

CHAPTER 2

CHAPTER 2: THE CONTRIBUTION OF MATERNAL CAFETERIA DIETS DURING PREGNANCY AND LACTATION TO BODY WEIGHT, FAT MASS AND GLUCOSE TOLERANCE IN THE OFFSPRING

2.1 INTRODUCTION

Obesity is currently a major public health issue across the developed and developing world [2, 3]. The dramatic rise in incidence of obesity in the population has led to a corresponding increase in the number of women entering pregnancy either overweight or obese [18, 19]. Maternal obesity is associated with an increased risk of pregnancy complications and is also an important risk factor for the later development of obesity in the child [21]. Experimental animal studies have suggested that this association is the result of exposure of the child to an increased nutrient supply during critical periods of development, which results in permanent alterations to the structure and function of key systems responsible for metabolic control [34-36].

More recently, there has been a growing interest in determining whether exposure to excess nutrition during different periods of development, specifically before birth and in the early postnatal period, has differential effects on the metabolic health of the offspring. However, the majority of studies to date which have investigated the consequences of maternal obesity on the metabolic health of the offspring have examined the impact of maternal obesity/maternal high-fat diets during both the prenatal and postnatal periods [15-17]. Furthermore, the few studies that have attempted to examine the separate contributions of prenatal and early postnatal exposure have produced conflicting results. Studies by Sun and colleagues demonstrated that rat pups who were cross-fostered onto dams consuming a high-fat diet at birth had increased adiposity and impaired glucose tolerance at 3 weeks of age in comparison with pups who were suckled by a control dam, and that this effect was independent of the nutritional

environment experienced before birth [106]. This suggested, therefore, that exposure to a high-fat diet during the early postnatal period was more important for metabolic programming than exposure before birth. Another study by Chang and colleagues [116], however, indicated that prenatal exposure to high-fat feeding was necessary and sufficient to program growth and fat mass in the offspring. Thus, the relative contribution of the prenatal and postnatal nutritional environment to the programming of obesity remains unclear.

Therefore, the aim of this Chapter was to utilise a cross-fostering approach in a rodent model to assess the relative contribution of exposure to maternal cafeteria diet during the prenatal and suckling periods on body weight, fat mass and glucose tolerance in the offspring at weaning and in early adolescence.

2.2 MATERIALS AND METHODS

2.2.1 Animals and feeding regime

This study was approved by the Animal Ethics Committee of the University of Adelaide. Twenty eight female Albino Wistar rats (200-250g) and four male Albino Wistar rats (200-300g) were used in this experiment. All rats were individually housed under a 12 hour light/12 hour dark cycle at a room temperature of 25^0C and allowed to acclimatise to the animal housing facility for at least one week before initiation of the experiment. During this time rats were fed *ad libitum* on standard rodent feed (Specialty Feeds, Glen Forrest, Western Australia, Australia) with free access to water.

At the end of the acclimatisation period, rats were randomly assigned to either the Control (n=15) or a Cafeteria (CAF; n=13) group. Control rats were given free access to standard rodent feed while CAF rats were fed a high-fat/high-sugar cafeteria diet comprised of peanut butter, hazelnut spread, chocolate-flavoured biscuits, extruded savoury snacks, sweetened multi-grain breakfast

cereal and a lard/rodent feed mix. Detailed nutritional composition of the cafeteria diet and control diet is shown in Table 2.1. Individual food intake was determined in all dams every two days before pregnancy, during pregnancy and lactation, and fresh food provided. All female rats were weighed once per week throughout the experiment.

Table 2.1 Nutritional details of cafeteria diet and standard rodent feed. Values for fat, carbohydrate and protein are expressed as a percentage of energy. All information was obtained from nutritional information provided by the manufacturer.

	Energy (kJ/g)	Fat (%en)	Carbohydrates (%en)	Protein (%en)	Sodium (mg/g)
Lard /rodent feed mix	20.9	19	51	17	3
Peanut Butter	25.5	48	18	25	4
Hazelnut Spread	23.6	37	54	5	4
Chocolate Biscuits	20.0	45	71	10	3
Savoury Snacks	23.1	34	55	6	10
Sweetened Cereal	16.4	2	86	6	5
Processed Meat	13.5	28	6	12	5
Standard rodent feed	18.0	5	60	20	4

2.2.2 Mating and pregnancy

After 4 to 6 weeks on their respective diets, vaginal smears were conducted daily on all females to determine their stage of estrous cycle. On the evening of diestrous/proestrous, two female rats were placed in a group cage with a male rat for 24 hours. Vaginal smears were performed the following morning to check for the presence of sperm in order to confirm successful mating and this was designated as gestation day 0. Female rats were then removed from the males and housed individually thereafter. Female rats were maintained on the same diet as before mating throughout pregnancy and lactation.

2.2.3 Cross-fostering

All dams were allowed to give birth naturally and all pups were born on day 21-22 of gestation. The number of pups in each litter and the sex and birth weight of each pup were measured on postnatal day 1(PND1). Within 24 hours of birth, all litters were culled to 8 pups, with 4 males and 4 females where possible. Blood and stomach contents were collected from the culled pups. Pups were then cross-fostered to another dam which gave birth within the same 24 hour period from either the same or different dietary treatment group. This resulted in 4 groups of offspring: litters from a Control dam cross-fostered onto another Control dam (Control-Control, C-C, n=9), litters from a Control dam cross-fostered onto a CAF dam (Control-CAF, C-CAF, n=6), litters from a CAF dam cross-fostered onto a Control dam (CAF-Control, CAF-C, n=6) and litters from a CAF dam cross-fostered onto another CAF dam (CAF-CAF, n=7). All the siblings were stayed together after cross-fostering and mixed litters were not used.

Pups remained with their foster mothers until weaning (3 weeks of age). After weaning, the pups were housed in groups with their same-sex littermates (3-4 pups/cage) and were fed with standard rat chow (Specialty Feeds, Glen Forrest, Western Australia, Australia) until 6 weeks of age. Pups were weighed every

second day until weaning and once per week thereafter until the end of the experiment.

2.2.4 Determination of glucose tolerance

Intraperitoneal glucose tolerance tests (IPGTT) were performed on 1 male and 1 female pup per litter after an overnight fast of ~18 hour at 6 weeks of age. Baseline blood samples were collected from the tail vein and a glucose bolus (2g/kg of 50% dextrose in sterile 0.9% saline) was then injected intraperitoneally. Blood samples were drawn from the tail vein at 5, 10, 15, 30, 60 and 120 min post glucose infusion. Blood glucose concentrations were tested on a calibrated handheld glucometer (Accu-Chek Performa©, Roche, Germany) using test reagent strips. Tests were performed in duplicate at each time point. The trapezoidal rule was used to determine the incremental area under the glucose curve (AUC) for all experimental animals.

2.2.5 Post-mortem and tissue collection

One male and one female pup from each litter were killed at weaning (3 weeks of age) and at 6 weeks of age and post-mortems were carried out to determine organ weights and body fat mass. The rats were not fasted before post-mortem and all post-mortems were conducted in light phase between 8 and 10 am. All rats were weighed immediately prior to post-mortem and were then killed with an overdose of CO_2. Blood samples were collected by cardiac puncture, and blood was centrifuged at 3,500g, 4^0C for 15 minutes and plasma stored at -20^0C for subsequent analysis of hormone and metabolite concentrations. Organs and individual fat depots including retroperitoneal fat, omental fat, gonadal fat, interscapular fat and subcutaneous fat were dissected and weighed. The weight of all major organs was recorded and a sample of all tissues was snap frozen in liquid nitrogen and stored at -80^0C.

2.2.6 Determination of plasma glucose and NEFA concentrations

Plasma glucose concentrations were determined using the Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA) which utilises the hexokinase/glucose-6-phosphate dehydrogenase method. This method converts glucose to 6-phosphogluconate (6-PG), NADH and hydrogen ions. The level of NADH formed is proportional to the amount of glucose in the sample and can be measured at an absorbance of 340nm. Plasma concentration of non-esterified free fatty acids (NEFA) was measured using the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). The kit utilises an enzymatic method which produces acyl-coenzyme A (CoA) from fatty acids by the addition of acyl-CoA synthetase (ACS). The acyl-CoA is oxidized by the addition of acyl-CoA oxidase (ACOD) with the generation of hydrogen peroxide and, in the presence of peroxidase (POD), this permits the oxidative condensation of 3-methy-N-ethyl-N(β-hydroxyethyl)-aniline (MEFA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm. These assays were conducted using the Konelab 20 system (Thermo Scientific, Vantaa, Finland). Inter and intra-assay coefficients of variation (CoV) for both glucose and NEFA assays were <5%.

2.2.7 Determination of plasma insulin and leptin concentrations

Plasma insulin concentrations were measured using a rat insulin enzyme immunoassay (EIA) (ALPCO Diagnostics, Salem, NH, USA) and leptin concentrations using a rat leptin enzyme-linked immunosorbent assay (ELISA) (Crystal Chem Inc., Downers Grove, IL, USA). Both assays utilised the sandwich type immunoassay method. Briefly, the plasma sample and anti-insulin or anti-leptin antibody conjugated with the horseradish peroxidase enzyme was added to a microplate previously coated with mouse anti-insulin antibody and rabbit anti-leptin antibody for insulin and leptin assays respectively. The plasma samples were then incubated to allow sufficient time for the binding of the antigen to the antibody. The excess unbound antibody was washed and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate added, which reacted with the

horseradish peroxidase (HRP) enzyme to give a blue colour. Stop solution (1N sulphuric acid) was added to stop the reaction. The microplate was then placed into the ELx808™ Absorbance Microplate Reader (BioTek Instruments Inc., VT) and the absorbance measured at 450nm with a reference wavelength of 620nm. The absorbance values at 450nm was normalised to the reference wavelength 620nm and subtracted from the absorbance of a blank sample to correct for non-specific background binding. The concentration of insulin or leptin in each plasma sample was determined from the standard curve using a 5-parameter logistic fit. Inter- and intra-assay CoV were <10%. All assays were conducted according to manufacturer's instructions with the exception of the modifications below.

2.2.7.1 *Validation of insulin and leptin ELISAs*

Prior to the actual experiment, linearity and spike-and-recovery tests were conducted to validate the assay for determining rat plasma insulin and leptin concentrations. The linearity test was conducted using serial dilutions of a plasma sample of known insulin/leptin concentrations. The concentrations of plasma hormones were determined and a linear curve plotted, with an r^2 of 0.995 - 0.999, indicating that there was a strong linear relationship between the concentration of leptin/insulin and the volume of plasma measured in the assay. The spike-and-recovery test was conducted to determine if there was a difference between the sample diluent and the biological sample matrix (i.e plasma) which could affect the ability of the assay to accurately detect the antigens (insulin and leptin). For this test, a known amount of a selected insulin/leptin standard was added to a plasma sample of known insulin/leptin concentration. The observed concentrations of plasma hormones were compared against the expected concentrations and the percentage of recovery calculated. The percentage of recovery was 90 - 100% for the insulin assay and 85 - 95% for leptin assay, both of which were within the recommended range for the respective assays.

2.2.7.2 *Variations from standard protocol*

Plasma samples with leptin concentrations which were above the highest standard provided in the kit were diluted with the sample diluent such that the concentration fell within the range of the standard curve (0.2 - 12.8ng/mL). The actual concentration of leptin in the sample was then determined by multiplying the concentration of the diluted samples by the dilution factor applied.

Plasma insulin concentrations in both male and female offspring at 3 weeks is not reported as the sample volume was insufficient to conduct the assay in a number of animals in each group and consequently there were not enough animal numbers in each group to produce meaningful results.

2.2.8 Statistical analyses

Data are presented as mean ± SEM. The effect of maternal cafeteria diet on maternal body weight, maternal intake of total energy, fat and carbohydrate before pregnancy, during pregnancy and lactation was determined using a Student's T-test. The effect of maternal cafeteria feeding on birth outcomes and birth weight was similarly determined. A Chi-squared test was used to compare the mortality rate of pups from Control and CAF pregnancies. The effect of maternal diet and sex on offspring body weight, body fat mass, plasma hormones and metabolite concentrations at 3 and 6 weeks of age were determined using a three-way ANOVA, with sex, prenatal and postnatal diet as factors. As three-way ANOVA revealed a large number differences and unequal variations between the sexes, the effect of prenatal vs postnatal environment and their interaction on body weight, body fat mass, plasma hormones and metabolite concentrations were determined separately in male and female offspring using a 2 way ANOVA for this and all subsequent Chapters. Where significant interactions were identified by ANOVA, the effect of the prenatal and postnatal exposure were analysed separately by Student's T-Test. Relationships between the plasma hormone and metabolite concentrations and total relative body fat mass was determined using linear regression analysis. The

multifactorial ANOVAs were performed using Stata 10 (Stata Corp LP, Texas, USA). All other analyses were performed using SPSS for Windows Version 17.0 (SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered statistically significant in all analyses. Grubb's tests were performed to identify the significant outliers in each group prior to the statistical analysis.

2.3 RESULTS

2.3.1 Maternal nutritional intake

2.3.1.1 *Nutritional intake of dams before pregnancy, during pregnancy and lactation*

Energy intake (KJ per day) was not different between the CAF and Control dams before pregnancy, but was higher in CAF dams during pregnancy (Figure 2.1B) and lower during lactation compared to Control dams (Figure 2.1C). Dams fed the high-fat, high-sugar cafeteria diet consumed more fat but less protein and carbohydrate compared to Control dams throughout the feeding period (Figure 2.1A, 2.1B, 2.1C).

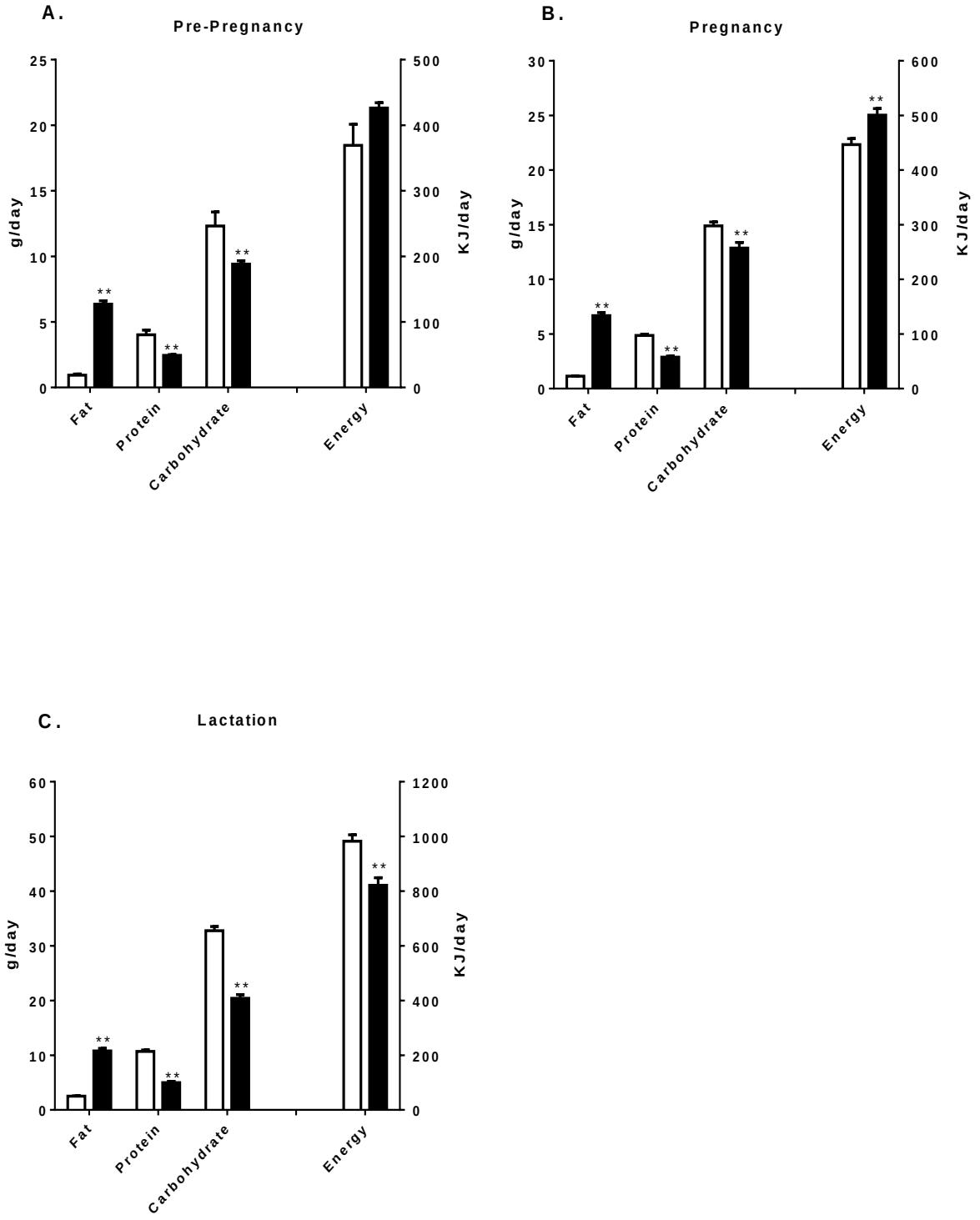


Figure 2.1 Mean daily intake of fat, protein, carbohydrate and total energy of Control dams (open bars) and CAF dams (solid bars) (A) before pregnancy, (B) during pregnancy and (C) during the lactation period. Values are expressed as mean ± SEM. ** denotes significance at *P<0.01*.

2.3.2 Maternal body weight

There was no difference in the average body weight of the Control and CAF dams before the cafeteria diet was introduced (Control = 257 ± 11.6g, CAF = 268 ± 17.6g). CAF dams were significantly heavier than Control dams at mating (Control = 315 ± 6.0g, CAF = 393 ± 8.6g, $P<0.01$), across pregnancy (Control = 363.38 ± 7.2g, CAF = 450.03 ± 9.5g, $P<0.01$) and across lactation (Control = 360.71 ± 4.1g, CAF = 427.86 ± 7.4g, $P<0.01$). CAF dams also gained more weight during pregnancy compared to Control dams (Control = 78.4 ± 9.3g, CAF = 113.4 ± 9.6g, $P<0.05$).

2.3.3 Birth and neonatal outcomes

There were no differences between Control and CAF dams in gestational age at delivery, litter size or the percentage of male and female pups (Table 2.2). However, mean body weights of pups at birth were lower in the CAF group (Table 2.2). There were no litters with dead pups in the Control group, but dead pups were found in 5/12 litters in the CAF group (Table 2.2) and a Chi-squared test indicated that there was a significant difference in pup mortality between the Control and CAF groups ($P<0.01$).

Table 2.2 Birth outcomes for Control and CAF pregnancies. Values are expressed as mean ± SEM. ** denotes significance at *P<0.01*.

	Control (n=15) Mean ± SEM	CAF (n=13) Mean ± SEM
Litter size (no. of pups)	13 ± 0.65	13 ± 0.68
Gestational age (days)	22 ± 0.10	22 ± 0.00
Birth weight (g)	7.15 ± 0.17	6.03 ± 0.13**
Live pups	13 ± 0.77	12 ± 0.65
Litters with dead pups	0%	42%**
Percent of male pups	47.7 ± 2.6	55.2 ± 3.7

2.3.4 Postnatal growth

2.3.4.1 *Suckling period (Growth to weaning)*

In both males and females, offspring born to CAF dams were lighter than offspring born to Control dams on postnatal day 2 (Male: C-C = 7.77 ± 0.33g, C-CAF = 7.55 ± 0.11g, CAF-C = 5.97 ± 0.24g, CAF-CAF = 6.34 ± 0.17g; *P<0.001*; Female: C-C = 7.45 ± 0.32g, C-CAF = 7.06 ± 0.08g, CAF-C = 5.91 ± 0.24g, CAF-CAF = 6.12 ± 0.16g; *P<0.001*). After this time, offspring suckled by CAF dams exhibited a slower growth rate than those suckled by a Control dam, and were lighter than offspring suckled by Control dams at weaning in both males and females, independent of whether the pups had been born to a Control or CAF dam (Figure 2.2). The male and female offspring in the C-CAF and CAF-CAF groups were also shorter at weaning compared to the CAF-C and C-C treatment groups (Male: C-C = 14.9 ± 0.2cm, CAF-C = 14.5 ± 0.5cm, C-CAF = 13.5 ± 0.1cm, CAF-CAF = 13.4 ± 0.3cm; *P<0.001*; Female: C-C = 14.3 ± 0.3cm, CAF-C = 14.2 ± 0.4cm, C-CAF = 13.2 ± 0.3cm, CAF-CAF = 13.3 ± 0.2cm; *P<0.01*). There was no difference in the abdominal circumference in the male offspring suckled by CAF dams compared to those suckled by Control dams at weaning. However the abdominal circumference at weaning in the female offspring suckled by CAF dams was significantly higher than those suckled by Control dams (Male: C-C = 10.7 ± 0.3cm, CAF-C = 10.3 ± 0.1cm, C-CAF = 10.2 ± 0.2cm, CAF-CAF = 10.4 ± 0.2cm; Female: C-C = 10.6 ± 0.1cm, CAF-C = 10.6 ± 0.3cm, C-CAF = 9.9 ± 0.4cm, CAF-CAF = 9.8 ± 0.2cm; *P<0.05).*

2.3.4.2 *Post-weaning period*

Offspring suckled by CAF dams remained lighter than the offspring suckled by Control dams after weaning, and were significantly lighter at 6 weeks of age in both males and females, independent of whether they were born to a Control or CAF dam (Figure 2.3). The female offspring suckled by CAF dams were also shorter than those suckled by Control dams (C-C = 21.2 ± 0.3cm, CAF-C = 20.6

± 0.3cm, C-CAF = 20.0 ± 0.3cm, CAF-CAF = 19.8 ± 0.4cm; *P<0.01*). In males, however, offspring born to CAF dams were shorter at 6 weeks of age compared to offspring born to Control dams, independent of whether they were suckled by a Control or CAF dam (C-C = 22.1 ± 0.3cm, CAF-C = 21.1 ± 0.3cm, C-CAF = 21.8 ± 0.1cm, CAF-CAF = 21.1 ± 0.3cm; *P<0.05*). The abdominal circumference in the male offspring suckled by CAF dams was not different from those suckled by Control dams at 6 weeks of age. However the abdominal circumference in the female offspring suckled by CAF dams remained significantly reduced compared to female offspring suckled by Control dams at 6 weeks of age (Male: C-C = 16.5 ± 0.4cm, CAF-C = 16.9 ± 0.5cm, C-CAF = 16.2 ± 0.2cm, CAF-CAF = 16.0 ± 0.3cm; Female: C-C = 15.5 ± 0.3cm, CAF-C = 15.1 ± 0.5cm, C-CAF = 14.8 ± 0.3cm, CAF-CAF = 14.4 ± 0.3cm; *P<0.05).*

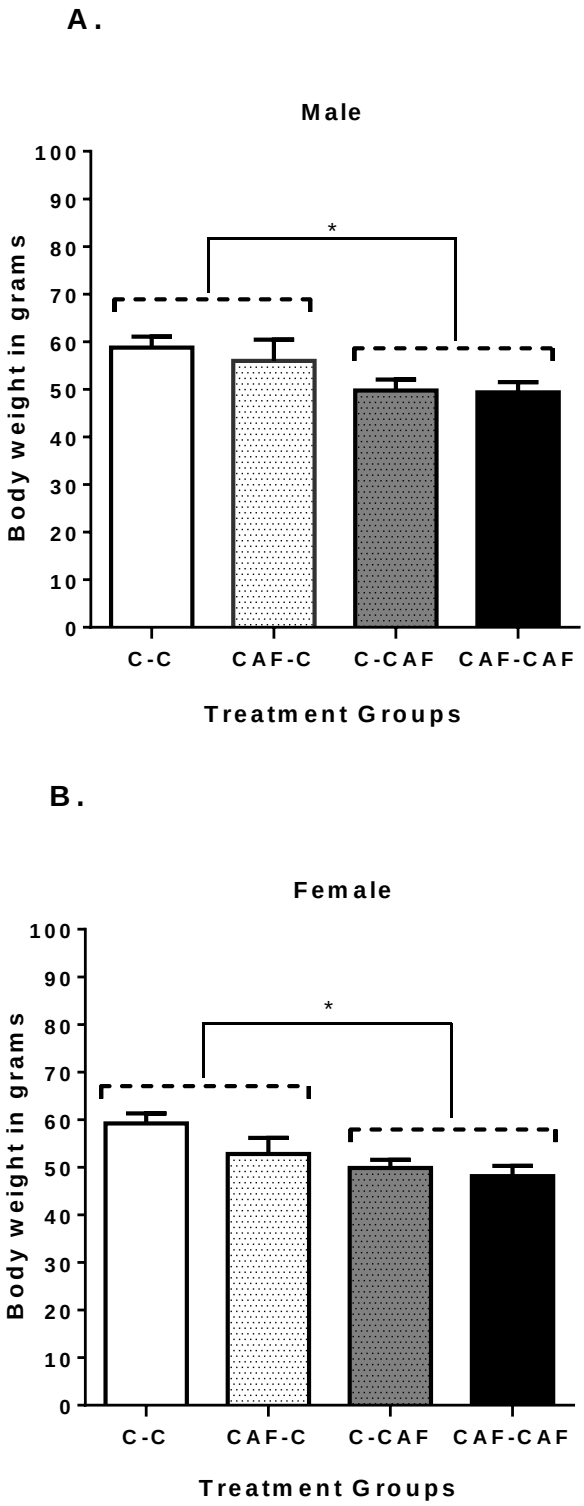


Figure 2.2 Body weight at weaning (3 weeks of age) for male (A) and female (B) offspring in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups. Values are expressed as mean ± SEM. * denotes significance at *P<0.05* and ** denotes significance at *P<0.01.*

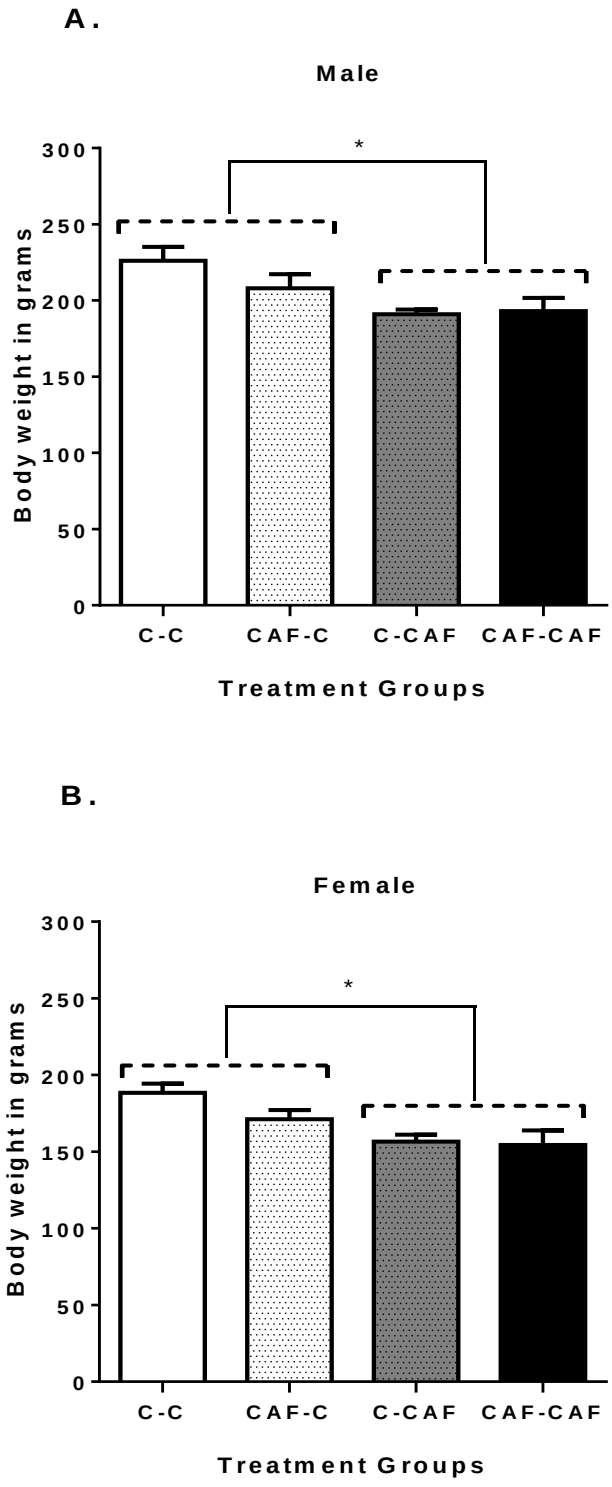


Figure 2.3 Body weight at 6 weeks of age (3 weeks after weaning) for male (A) and female (B) offspring in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups. Values are expressed as mean ± SEM. * denotes significance at *P<0.05* and ** denotes significance *at P<0.01*.

2.3.5 Effect of prenatal and postnatal nutritional exposure on offspring body composition at 3 weeks and at 6 weeks of age

2.3.5.1 *3 Weeks*

At 3 weeks of age, both male and female offspring suckled by a CAF dam had a higher total percentage body fat compared to those suckled by a Control dam (Figure 2.4). The relative mass of individual fat depots including that gonadal fat, interscapular fat, retroperitoneal fat, omental fat and subcutaneous fat were all higher in offspring suckled by a CAF dam (C-CAF and CAF-CAF) compared to offspring suckled by a Control dam (C-C and CAF-C) in both males and females (Table 2.3) and the fat deposition was mainly in the subcutaneous fat depot (data not shown). There were no differences between the groups in relative weight of other major organs except the brain in male offspring, which was higher in offspring suckled by a CAF dam compared to those suckled by a Control Dam (Table 2.3). These effects were all independent of whether pups had been born to a control or CAF dam.

2.3.5.2 *6 Weeks*

There was no difference in percentage total body fat between any of the treatment groups in either males or females at 6 weeks of age (Figure 2.5). However, there were differences in the weights of individual fat depots. In females, but not in males, the mass of the interscapular fat depot as a percentage of body weight was higher in offspring suckled by CAF dams compared to the female offspring suckled by Control dams independent of whether they were born to a Control or CAF dam (Table 2.4). There were no differences between the groups in the relative weight of other major organs at 6 weeks of age except the relative weight of lung in males and the relative weight of heart in females, which were higher in the offspring suckled by CAF dams compared to offspring suckled by Control dams (Table 2.4).

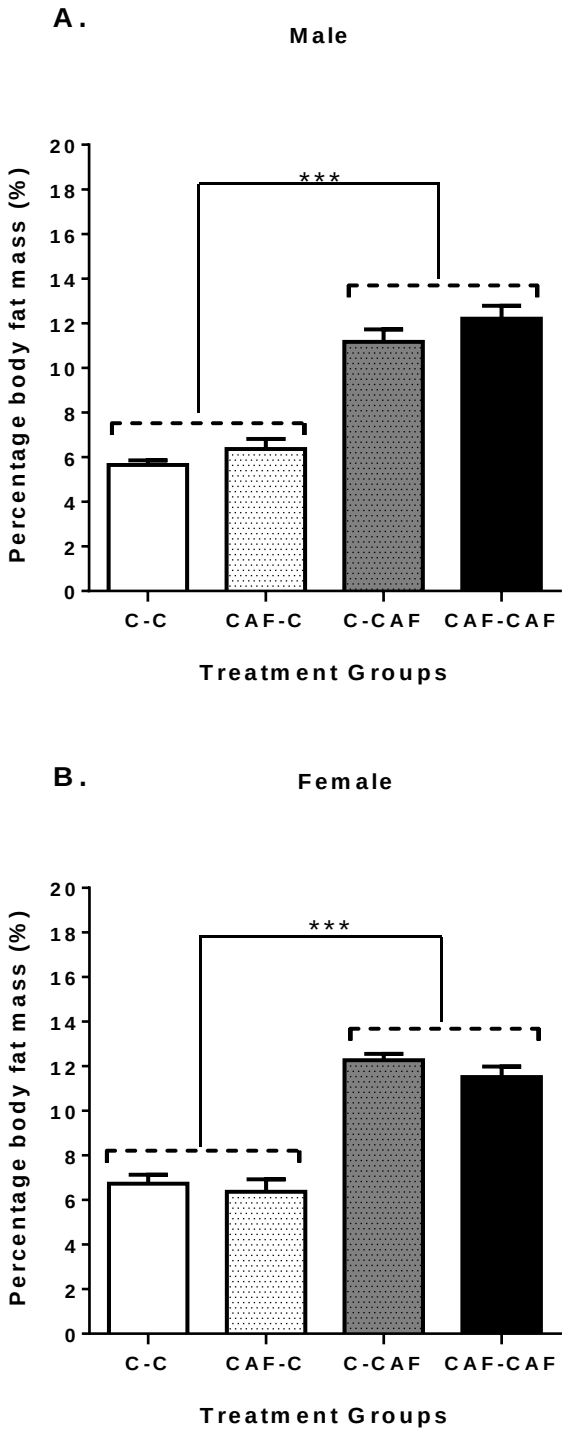


Figure 2.4 Body fat mass (expressed as a percentage of total body weight) in male (A) and female (B) offspring in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups at 3 weeks of age. Values are expressed as mean ± SEM. *** denotes significance at *P<0.001*.

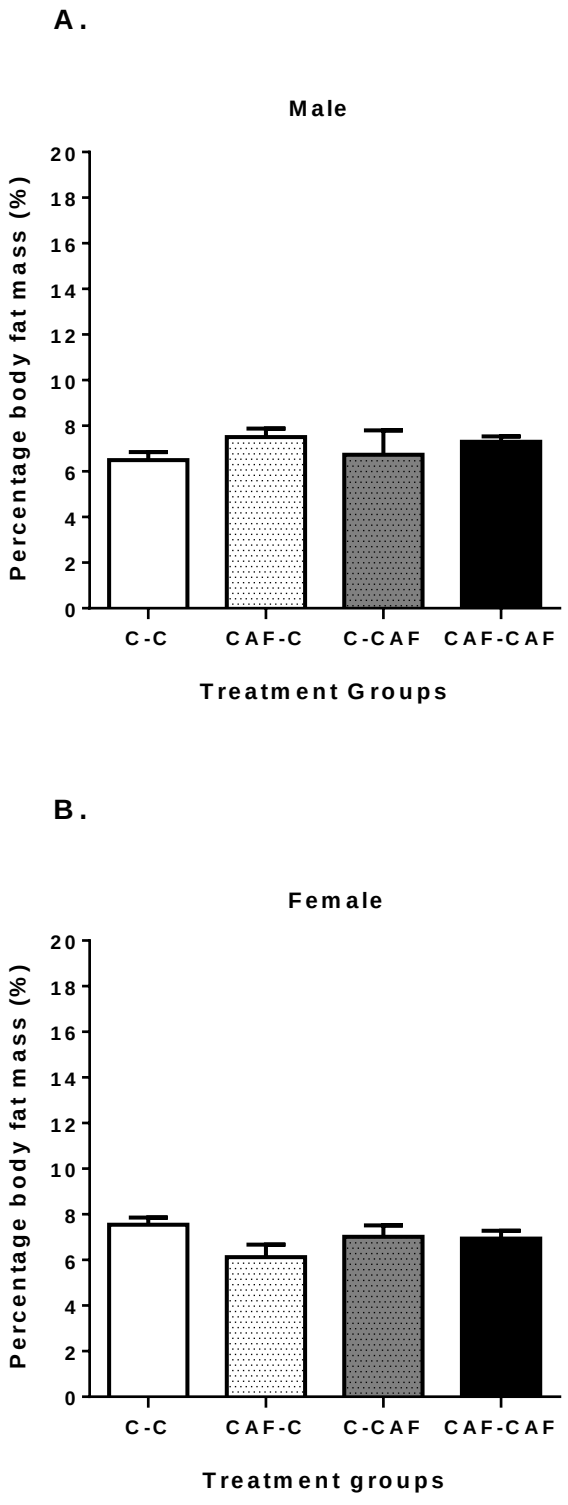


Figure 2.5 Body fat mass (expressed as a percentage of total body weight) in male (A) and female (B) offspring in the C-C (open bars), C-CAF (solid bars with pattern), CAF-C (open bars with pattern) and CAF-CAF (solid bars) groups 6 weeks of age. Values are expressed as mean ± SEM.

Table 2.3 Mass of individual fat depots and major organs expressed as a percentage of bodyweight in male and female offspring in the C-C, CAF-C, C-CAF and CAF-CAF groups at 3 weeks of age. Values are expressed as means ± SEM. * denotes significance at *P<0.05, ** denotes significance at P<0.01* and *** denotes significance at *P<0.001.*

Parameter	Male				Female			
	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Gonadal fat	0.19±0.02	0.20±0.02	0.37±0.05***	0.38±0.04***	0.27±0.03	0.24±0.03	0.61±0.05***	0.61±0.06***
Interscapular fat	0.63±0.03	0.59±0.04	1.04±0.11**	0.86±0.10**	0.64±0.04	0.69±0.03	0.90±0.04***	0.84±0.04***
Retroperitoneal	0.40±0.02	0.40±0.07	0.76±0.05***	0.96±0.07***	0.43±0.02	0.33±0.02	0.75±0.05***	0.72±0.02***
Omental fat	0.55±0.03	0.51±0.06	0.77±0.06***	0.73±0.05***	0.48±0.03	0.55±0.04	0.65±0.02***	0.78±0.04***
Subcutaneous fat	3.88±0.18	4.66±0.35	8.22±0.58***	9.27±0.57***	4.92±0.36	4.55±0.49	9.36±0.23***	8.57±0.47***
Brain	2.52±0.06	2.76±0.16	2.91±0.19*	2.92±0.11*	2.58±0.07	2.79±0.26	2.75±0.10	2.90±0.12
Liver	3.96±0.13	3.94±0.11	3.82±0.16	3.96±0.16	4.26±0.10	3.95±0.09	3.88±0.11	3.89±0.16
Lung	0.96±0.06	0.90±0.03	0.92±0.03	0.88±0.04	1.00±0.06	1.04±0.23	1.05±0.08	0.81±0.06
Pancreas	0.37±0.03	0.41±0.05	0.35±0.05	0.25±0.04	0.42±0.03	0.37±0.09	0.41±0.04	0.35±0.03
Heart	0.52±0.02	0.58±0.02	0.60±0.03	0.58±0.03	0.55±0.01	0.56±0.06	0.58±0.04	0.58±0.02

Table 2.4 Mass of individual fat depots and major organs expressed as a percentage of body weight in male and female offspring in the C-C, CAF-C, C-CAF and CAF-CAF groups at 6 weeks of age. Values are expressed as means ± SEM. * denotes significance at *P<0.05* and ** denotes significance at *P<0.01*.

Parameter	Male				Female			
	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Gonadal fat	0.67±0.05	0.74±0.10	0.72±0.04	0.69±0.02	0.97±0.07	0.80±0.13	0.77±0.08	0.77±0.12
Interscapular fat	0.31±0.03	0.34±0.05	0.39±0.03	0.36±0.02	0.34±0.02	0.31±0.01	0.37±0.03*	0.38±0.02*
Retroperitoneal	0.74±0.09	0.87±0.08	0.86±0.06	0.87±0.06	0.77±0.06	0.64±0.05	0.72±0.06	0.80±0.11
Omental fat	0.71±0.05	0.68±0.04	0.74±0.03	0.74±0.07	0.84±0.03	0.64±0.07	0.69±0.05	0.68±0.07
Subcutaneous fat	4.08±0.24	4.88±0.21	4.02 ±1.09	4.63±0.14	4.63±0.28	3.73±0.36	4.48±0.36	4.31±0.18
Brain	0.88 ±0.03	0.89±0.03	0.94±0.01	0.95±0.03	0.97±0.02	1.07±0.04	1.08±0.04	1.10±0.05
Liver	4.57±0.14	4.47±0.13	4.69±0.21	4.61±0.05	4.61±0.16	4.79±0.20	4.84±0.15	4.55±0.12
Lung	0.65±0.03	0.58±0.04	1.02±0.12**	0.72±0.07**	0.82±0.07	0.72±0.03	0.74±0.04	0.80±0.03
Pancreas	0.34±0.03	0.41±0.09	0.34±0.02	0.36±0.04	0.38±0.04	0.44±0.05	0.42±0.07	0.40±0.03
Heart	0.43±0.02	0.41±0.02	0.44±0.04	0.44±0.02	0.43±0.01	0.42±0.01	0.43±0.02*	0.47±0.02*

2.3.6 Effect of prenatal and postnatal nutritional exposure on offspring plasma hormone and metabolite concentrations at 3 weeks and 6 weeks of age

At 3 weeks of age, plasma leptin concentrations were higher in offspring suckled by CAF dams compared to offspring suckled by Control dams in both males and females (Table 2.5). However, at 6 weeks of age, female offspring born to Control dams had higher plasma leptin concentrations compared to female offspring born to CAF dams independent of their exposure during the suckling period (Table 2.5). There was no difference in plasma leptin concentrations between the groups at 6 weeks of age in males (Table 2.5).

Plasma glucose concentrations were not different between the groups in male offspring at either 3 weeks or 6 weeks of age (Table 2.5). In females, however, offspring born to CAF dams had higher plasma glucose concentrations at 3 weeks of age compared to offspring born to Control dams, independent of their nutritional exposure during the suckling period (Table 2.5). There were no differences in plasma glucose concentrations between groups in females at 6 weeks of age. Plasma NEFA concentrations were not different between the groups in males or females at either 3 weeks or 6 weeks of age (Table 2.5). Plasma insulin concentrations were also not different between the groups in either males or females at 6 weeks of age (Table 2.5).

Table 2.5 Plasma concentrations of glucose, NEFA and leptin in male and female offspring in the C-C, C-CAF, CAF-C and CAF-CAF groups at 3 weeks and plasma concentrations of glucose, NEFA, insulin and leptin in male and female offspring in the C-C, C-CAF, CAF-C and CAF-CAF groups at 6 weeks of age. Values are expressed as means ± SEM. * denotes significance at *P<0.05* and ** denotes significance at *P<0.01.*

3 Weeks

Parameter	Male				Female			
	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Glucose (mmol/L)	13.01±1.34	13.68±0.91	14.23±1.79	14.35± 2.00	10.02±0.51	13.35±1.28*	12.15±1.04	13.71±1.28*
NEFA(µEq/L)	0.47±0.10	0.57±0.09	0.59±0.07	0.46 ±0.11	0.45±0.10	0.65±0.10	0.65±0.15	0.63±0.08
Leptin (ng/ml)	5.68±0.87	5.84±1.85	7.85±2.40**	15.38±1.59**	5.84±0.74	6.49±1.39	10.56±1.92**	13.77±1.81**

6 Weeks

Parameter	Male				Female			
	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Glucose (mmol/L)	17.71±1.50	15.15±1.78	16.91±2.88	13.77±0.93	18.31±2.55	13.49±0.44	14.59±1.12	14.57±0.98
NEFA(µEq/L)	0.61±0.07	0.53±0.07	0.60±0.04	0.65±0.12	0.73±0.05	0.51±0.08	0.63±0.10	0.69±0.10
Insulin (ng/ml)	0.98±0.29	0.23±0.01	0.37±0.29	0.89±0.52	1.05±0.32	0.72±0.37	0.44±0.07	1.49±0.54
Leptin (ng/ml)	5.04±0.47	5.97±0.87	6.05±0.72	5.78±0.43	5.03±0.36*	3.52±0.51	5.14±0.44*	4.20±0.49

2.3.7 Relationship between plasma hormone and metabolite concentrations and total relative fat mass and individual fat mass at 3 weeks and 6 weeks of age

When data from all treatment groups were combined, plasma leptin concentrations at 3 weeks of age were directly correlated with total fat mass and mass of gonadal fat, retroperitoneal fat, omental fat and subcutaneous fat relative to body weight in both males and females (Table 2.6). Plasma leptin concentrations were also positively correlated with interscapular fat mass in females, and tended (*P=0.07*) to be positively correlated with interscapular fat mass in males at 3 weeks of age (Table 2.6). Plasma leptin concentrations were also positively correlated with total body fat mass, retroperitoneal fat and subcutaneous fat mass relative to body weight in females at 6 weeks of age, independent of treatment group (Table 2.6). Plasma leptin concentrations at 6 weeks of age also tended to be positively correlated with retroperitoneal fat mass in males (*P=0.07*) and omental fat mass (*P=0.06)* in females (Table 2.6).

There was no significant relationship between plasma glucose or NEFA concentrations and total relative fat mass or mass of gonadal fat, interscapular fat, retroperitoneal fat or omental fat in either males or females at 3 weeks of age. However, plasma glucose and NEFA concentrations at 3 weeks tended to be positively correlated with subcutaneous fat mass relative to body weight in females (glucose, *P=0.07*; NEFA, *P=0.06*, Table 2.6), but not in males, independent of treatment group. At 6 weeks of age, however, plasma glucose concentrations in females, but not in males, were positively correlated with subcutaneous fat mass and plasma glucose concentrations in males, but not in females, were positively correlated with interscapular fat relative to body weight, independent of treatment group (Table 2.6).

There was a significant positive relationship between plasma insulin concentrations and total relative fat mass and the relative weight of all the individual fat depots in male, but not female offspring at 3 weeks of age (Table 2.6). There was no relationship between insulin concentrations and total relative

fat mass and mass of individual fat depots in males at 6 weeks of age (Table 2.6). In females, however, there was a significant positive relationship between plasma insulin concentrations and the relative mass of the retroperitoneal fat depot at this time point (Table 2.6). There was also no relationship between plasma insulin concentrations and total relative fat mass or mass of other individual fat depots at 6 weeks of age (Table 2.6).

2.3.8 Glucose tolerance

There were no significant differences in glucose tolerance at 6 weeks of age as assessed by the Glucose AUC following an IP glucose challenge in either male or female offspring (Male, $glucose_{AUC}$: C-C = 1140.2 ± 56.8, CAF-C = 1155.8 ± 58.3, C-CAF = 1227.4 ± 57.7, CAF-CAF = 1025.9 ± 116.0; Female, $glucose_{AUC}$: C-C = 1111.0 ± 37.1, CAF-C = 1155.0 ± 98.9, C-CAF = 1201.2 ± 42.1, CAF-CAF = 1122.9 ± 67.1; Figure 2.6).

Table 2.6 The relationship between percentage total body fat mass and percentage individual fat masses with plasma glucose, NEFA, insulin and leptin concentrations, independent of treatment groups, in the male and female offspring at 3 weeks and 6 weeks of age.

3 weeks

	Glucose		NEFA		Insulin		Leptin	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Total body fat mass	ns	ns	ns	ns	r^2= 0.832, *P<0.001*	ns	r^2= 0.553, *P<0.001*	r^2= 0.410, *P<0.01*
Gonadal fat	ns	ns	ns	ns	r^2= 0.607, *P<0.01*	ns	r^2= 0.253, *P<0.05*	r^2= 0.301, *P<0.05*
Interscapular fat	ns	ns	ns	ns	r^2= 0.413, *P<0.05*	ns	r^2= 0.163, *P=0.07*	r^2= 0.210, *P<0.05*
Retroperitoneal fat	ns	ns	ns	ns	r^2= 0.685, *P<0.01*	ns	r^2= 0.445, *P<0.01*	r^2= 0.407, *P<0.01*
Omental fat	ns	ns	ns	ns	r^2= 0.355, *P=0.05*	ns	r^2= 0.210, *P<0.05*	r^2= 0.406, *P<0.01*
Subcutaneous fat	ns	r^2= 0.168, *P= 0.07*	ns	r^2= 0.189, *P=0.06*	r^2= 0.828, *P<0.001*	ns	r^2= 0.575, P<0.001	r^2= 0.383, *P<0.01*
6 weeks								
Total body fat mass	ns	ns	ns	ns	ns	ns	ns	r^2= 0.383, *P<0.01*
Gonadal fat	r^2= -0.162, *P=0.07*	ns	r^2= -0.162, *P=0.07*	ns	ns	ns	ns	ns
Interscapular fat	r^2= 0.362, *P<0.01*	ns	ns	ns	ns	ns	ns	ns
Retroperitoneal fat	ns	ns	ns	ns	ns	r^2= 0.451, *P<0.001*	r^2= 0.174, *P=0.07*	r^2= 0.207, *P<0.05*
Omental fat	ns	ns	ns	ns	ns	ns	ns	r^2= 0.147, *P=0.06*
Subcutaneous fat	ns	r^2= 0.166, *P<0.05*	ns	ns	ns	ns	ns	r^2= 0.356, *P<0.01*

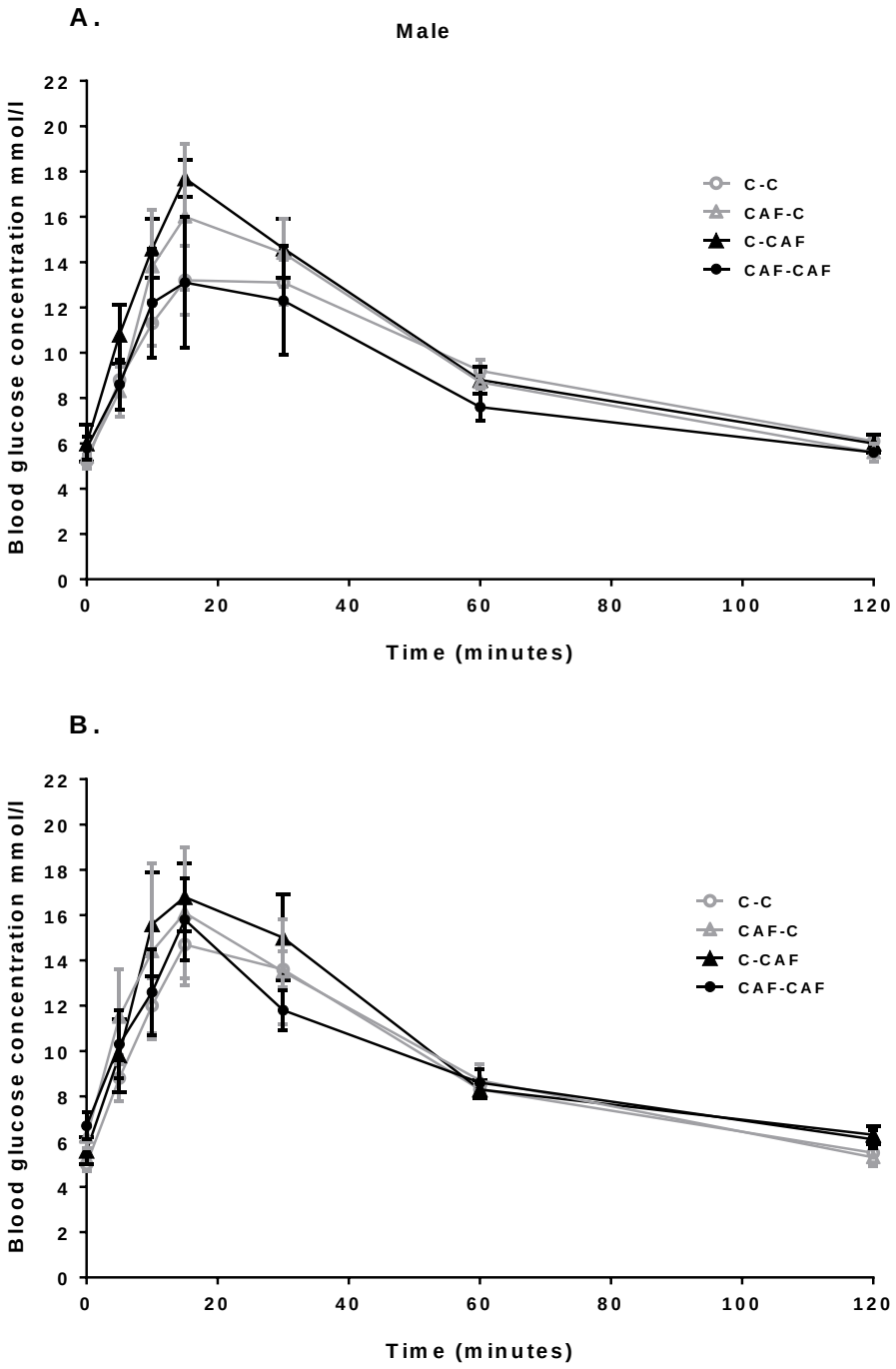


Figure 2.6 Blood glucose concentrations during the 2 hour glucose tolerance test (2.0g/kg, intraperitoneal injection) results in male (A) and female (B) offspring at 6 weeks of age. The incremental area under the glucose curve (AUC) for all experimental animals was determined using the trapezoidal method. Open grey circles: C-C group, Open grey triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group. Values are expressed as mean ± SEM.

2.4 DISCUSSION

In this chapter, I have demonstrated that exposure to high-fat cafeteria diet during the suckling period is more important for determining growth rate after birth and body fat mass at weaning than exposure before birth. Importantly, the fact that increased body fat at weaning was not increased in offspring born to CAF fed dams who were suckled by a Control dam provided evidence that the adverse effects of early exposure to high-fat cafeteria diets could potentially be reversed by early postnatal nutritional interventions. Although several models of maternal high-fat feeding have demonstrated the association between maternal and child obesity [17, 408], the present study is one of only few studies which has separated the effects of being exposed to a maternal high-fat/high-sugar cafeteria diet before birth from those of being exposed during the suckling period.

2.4.1 Birth and neonatal outcomes

Although gestational age at delivery and total litter size was not different between Control and CAF dams, the CAF group exhibited significantly poorer birth outcomes, as indicated by a greater number of pups that did not survive and a reduced birth weight in live born pups. The greater pup mortality observed in CAF dams is in line with previous clinical studies which have shown that maternal obesity and excessive weight gain during pregnancy leads to increased fetal mortality rates [409, 410]. In this study I was not present for all deliveries, and cannot be sure whether the pups later discovered to be dead were stillborn, or died after birth. It is also possible, therefore, that the high-fat/high-sugar cafeteria diet affected dam behaviour in a way which impacted on pup survival, and a previous study suggested that maternal high-fat feeding was associated with higher levels of maternal cannibalism [411]. However, maternal behaviour was not specifically assessed in this study and the reason for the increased rate of pup death remains unclear.

I also found that the mean body weight of pups at birth was significantly reduced in the CAF dams. Maternal high-fat feeding in rodent models has been reported to have conflicting effects on birth weight, with some studies reporting no effects [16, 102, 103, 107] while others report either decreased [93, 224, 412, 413] or increased birth weights [17]. These disagreements are likely to be due to the difference in the composition of the fat enriched diets across studies with variable levels of maternal energy and macronutrient intakes [17, 408]. The CAF dams in the present study had increased energy and fat intakes but reduced protein intake during pregnancy, and it is possible that the low protein intake of dams during gestation may have been the most important factor contributing to the reduced pup weight, since this would be expected to limit the supply of essential amino acids for tissue accretion in the offspring [414]. It has been established in a number of previous studies that maternal consumption of low-protein diets during gestation results in reduced fetal growth and low birth weight in the offspring [87, 88, 415-418]. It is also possible that the growth restriction may relate to maternal and placental adaptations to high-fat feeding and there is evidence from animal studies that maternal obesity in pregnancy is associated with an increased risk of placental dysfunction, which would inhibit the supply of oxygen and nutrients to the fetus and therefore reduce growth, however this was not directly assessed in the present study [419].

2.4.2 Postnatal growth

Growth from birth to weaning was significantly affected by the maternal diet during the lactation period, and those pups who were suckled by CAF dams had a lower body weight at weaning, independent of whether they had had a normal (pups born to Control dams) or low (pups born to CAF dams) weight at birth. Importantly, it should be noted that growth was restored in pups born to CAF dams who were suckled by Control mothers, suggesting that the growth deficits and macro/micronutrient deficiencies reported by previous studies due to high-fat/high-sugar feeding during pregnancy [163] can be overcome by restoring appropriate nutrition during the lactation period.

There have been contradictory results from previous studies in relation to the effect of maternal high-fat diets on postnatal weight gain, and whilst the majority of these studies have reported an increase in body weight throughout the life course [16, 420], a number of more recent studies have reported either no difference or reduced body weights at weaning in the offspring of high-fat fed dams [101, 117, 421]. Variations in the composition of the maternal high-fat diet and the genetic background of the strain under investigation are likely to account for much of this discrepancy, but further studies are required to fully define the mechanisms involved.

Although CAF dams consumed less energy and less protein than Control dams during lactation in our study, the lower maternal energy intake cannot entirely explain the reduced growth rate of the pups, since previous studies have reported that the energy intake of the dams during lactation is not related to pup growth [422]. However, previous studies have reported that maternal obesity is associated with impaired lactation performance in both humans and animals [423-426], suggesting that the reduced growth rate during the suckling period may have been a result of a reduced milk supply to the pups. In addition, it has also been demonstrated in previous studies that a decrease in maternal protein intake significantly inhibits milk production and this may also have decreased the volume of milk available to support the growth of the pups suckled by CAF dams [109, 427-431]. However, protein intake is unlikely to be the only factor affecting lactation performance of CAF dams, since other studies have reported that increasing the protein intake of dams consuming a cafeteria diet to the levels in the control rat feed during the lactation period, did not normalise pup growth [196]. It is also possible that the high fat content of the diet is responsible for the poor lactation performance of CAF dams since it has been observed that high fat consumption negatively affects mammary gland lipogenesis and therefore energy content of the milk [432]. In light of the findings of the present study, studies which directly assess milk production in control and cafeteria-fed dams are clearly warranted.

In addition to effects on milk production, it is also possible that the cafeteria diet/higher maternal body weight had a negative effect on the dams nursing behaviour. Two previous studies have reported a reduction in total nursing time in obese dams [422, 433] and whilst I did not observe any obvious difference in maternal care between the feeding groups in the present study, I did not quantitatively asses this.

I also observed the growth deficits in pups suckled by CAF dams were maintained at 6 weeks of age, even when these animals were weaned onto a nutritionally complete rodent chow. This adds to evidence from previous studies that maternal nutrition during pregnancy can exert long-lasting effects on body weight and growth potential of the offspring [41]. By way of example, global caloric restriction in rats before and during pregnancy results in permanent growth stunting of the offspring [434, 435], and maternal protein restriction during both pregnancy and lactation is associated with growth restriction in the offspring that persists until adulthood [436]. The observations from the present study extend these findings to suggest that exposure to a maternal cafeteria diet, low in protein and micronutrient density, during the suckling period alone results in deficits in pup growth, that persist even when they are weaned onto a nutritionally complete diet. The findings from the present study also provide evidence that in rodents, the maternal diet during lactation is more important for determining the postnatal growth of pups than the maternal diet during pregnancy.

2.4.3 Body fat mass

The results of this chapter demonstrate that the exposure to high-fat/high-sugar diet during the suckling period resulted in increased body fat mass in the offspring at weaning, independent of whether or not they were exposed to maternal high-fat/high-sugar feeding before birth. This finding was in line with the outcomes of a previous study in which pups cross-fostered to high-fat dams exhibited higher adiposity at weaning than those cross-fostered to Control dams

[106]. Importantly, in the present study, when the offspring born to Control dams were exposed to CAF dam's milk during the suckling period, they had a higher body fat accumulation at weaning compared to either Control or CAF offspring suckled by Control dams. It could also be that the pups were eating some of their mother's high-fat diet towards the end of suckling period, which would have contributed to increased fat deposition. Conversely, offspring born to CAF dams had reduced body fat mass at weaning when they were suckled by a Control dam compared to CAF offspring who were cross-fostered onto another CAF dam. This strongly implicates the nutritional environment during the suckling period as being more important than that during the prenatal period in determining body fat accumulation in the offspring during early postnatal life.

The suckling period has been identified as a critical time window for fat deposition in rodents, since rats and mice deposit very little fat before birth. The postnatal nutritional environment in rodents is so critical that it can even override genetic predisposing factors in the development of obesity phenotype [107]. Unlike rats, however, human infants deposit significant fat stores before birth, and it is not possible to directly extrapolate the results from rodent studies to humans. The results of this study do, however, raise the possibility that improving the quality of the maternal diet later in pregnancy in a human context could potentially reduce the risk of excess fat deposition in infants who are exposed to an excess nutrient supply earlier in gestation.

One of the key findings of this study was that providing a nutritionally balanced diet after weaning could reverse the increase in fat deposition induced by exposure to the cafeteria diet earlier in development. Although previous studies [107, 142] have demonstrated that alterations in the postnatal environment can override some of the influences of prenatal and genetic factors in determining the development of obesity in offspring, these studies didn't assess the effect of a healthy diet on fat deposition and on other metabolic outcomes after weaning. The present study provides further evidence that providing a nutritionally balanced diet post-weaning can reverse the negative effects of exposure to a

high-fat/high-sugar earlier in postnatal life. While total body fat mass was no longer different between groups at 6 weeks of age, the relative weights of some of the individual fat depots, for example the interscapular depot, were still significantly higher at this time in offspring suckled by CAF dams. The reason for this is not entirely clear, and further studies are required to determine if this effect persists into adulthood, and if it has any impact on overall metabolic health, particularly in light of the fact that the interscapular depot is comprised mainly of brown adipose tissue [437].

2.4.4 Plasma leptin and insulin concentrations

In the present study, I observed that plasma leptin concentrations at weaning were significantly higher in offspring suckled by a CAF dam in both males and females compared to offspring suckled by Control dams, in line with the increase in percentage body fat mass in this treatment group. These results are consistent with a number of previous studies showing that plasma leptin concentrations are increased in overweight and obese humans compared to lean individuals and are positively correlated with BMI and percentage body fat in both animals and humans [438, 439]. Interestingly, plasma leptin concentrations in males at 6 weeks of age were no longer correlated with relative body fat mass or individual fat depots except a trend towards a positive relationship with relative retroperitoneal fat mass. It is possible that this may indicate an altered relationship between fat storage and leptin synthesis in the fat depots of these offspring; however this remains to be confirmed on a molecular level. In all groups of animals, plasma leptin concentrations were lower at 6 weeks than at 3 weeks of age. This observation is consistent with previous studies [344] and is likely to be due to the switch from a high-fat milk to a high-carbohydrate diet at weaning. I also observed that plasma insulin concentrations at weaning were positively correlated to total relative body fat mass in the males, but not in females. It has been established in previous rodent studies that males are more sensitive to the anabolic actions of insulin compared to females [440]. It has been also reported that elevated insulin levels

observed in male rats in response to chronic high-fat intake was not observed in females [441], consistent with the present observations, and suggesting a sex-dependent regulation of both fat deposition and glucose homeostasis.

2.4.5 Plasma glucose and glucose tolerance

I found that female offspring exposed to the cafeteria diet before birth had significantly elevated plasma glucose concentrations at weaning independent of whether they were suckled by a control or CAF dam. It has been reported previously [400] that a chronic exposure to a high-fat diet during pregnancy, lactation and post-weaning periods significantly increased the plasma glucose concentrations in the female offspring compared to those who were exposed to control or high-fat diet during the perinatal period and were given the opposite diet after weaning. This previous study did not assess the separate effects of prenatal and postnatal nutritional exposure on offspring metabolic health. They did, however, report that female offspring exposed to a high-fat diet were more hyperglycemic than males, which is consistent with the present study and others and implies that the effect of maternal diet during pregnancy and lactation on offspring glucose metabolism may be sex-specific [442]. However, it remains unclear what mechanisms are responsible for these sex differences or why exposure to maternal cafeteria nutrition had no effect on the plasma glucose concentration in the male offspring. It is also important to note that the blood samples in the present study were not collected in the fasting state, so it is not possible to draw any clear conclusions, and further studies are needed to be able to properly interpret this finding.

I found no effect of exposure to a cafeteria diet either before birth or during the suckling period on glucose tolerance at 6 weeks of age. This was unexpected given previous cross-fostering studies which have reported significant reductions in glucose tolerance in both male and female offspring after exposure to a high-fat diet/maternal obesity during either prenatal or postnatal period [106, 443]. One possibility is that weaning the offspring to a standard chow reversed the impact of exposure to a cafeteria diet before birth and/or during the suckling

period on glucose tolerance, and it will be important to assess glucose tolerance at weaning in future studies.

2.5 SUMMARY

In summary, I have demonstrated that exposure to high-fat cafeteria diet during the suckling period results in increased fat mass in the offspring independent of whether they were born to a dam consuming a control or high-fat cafeteria diet. Thus, the findings from the present study suggest that exposure to maternal high-fat feeding during the suckling period is more important for determining postnatal growth and body fat mass at weaning than exposure to high-fat diet before birth. The results of this Chapter also provide evidence that a nutritionally balanced post-weaning diet can reverse the increase in fat deposition induced by being exposed to a high-fat diet during the perinatal period although it remains to be determined if this effect persists at older ages. In the subsequent Chapters, I aimed to determine the potential mechanisms through which maternal cafeteria diets during lactation promote body fat accumulation in the offspring, by examining the impact of the cafeteria diet on the composition of the maternal milk (Chapter 3) and on the expression of key adipogenic and lipogeneic genes in the offspring at weaning and at 6 weeks of age (Chapter 4). Finally, I aimed to determine the relative impact of exposure to the maternal cafeteria diet during the prenatal and suckling periods on the susceptibility of the pups to diet-induced obesity in young adulthood (Chapter 5).

CHAPTER 3

CHAPTER 3: EFFECT OF A 'CAFETERIA DIET' ON MATERNAL MILK AND OFFSPRING RED BLOOD CELL FATTY ACID COMPOSITION AND ITS RELATIONSHIP TO OFFSPRING FAT MASS

3.1 INTRODUCTION

Epidemiological, clinical and experimental animal studies have shown that maternal obesity, maternal hyperglycemia or maternal intake of diets high in saturated fat, sugar or total calories during pregnancy and lactation is associated with an increased risk of obesity and metabolic disease in the offspring in postnatal life [399, 400]. More recently, studies have attempted to isolate the separate contributions of prenatal and postnatal nutritional exposures on subsequent metabolic outcomes, and these studies have demonstrated that exposure to maternal cafeteria diets during the lactation period alone produces comparable detrimental effects to offspring metabolic health to those resulting from exposure during the entire perinatal period [106, 107]. In Chapter 2, I reported that the fat mass of offspring at weaning was significantly increased in offspring suckled by a dam consuming a cafeteria diet, irrespective of whether they were born to a Control or Cafeteria fed dam. Since maternal milk is the dominant source of nutrition for the offspring during the suckling period, these findings suggest that alterations to milk composition as a result of inappropriate maternal nutrition may play a central role both in driving the increased adiposity of these offspring at weaning and in metabolic programming.

The diets used in the majority of studies focused on the adverse consequences of maternal obesogenic diets, including that used in Chapter 2 of this thesis, consist of a selection of palatable foods, and typically contain a higher proportion of total fat, sugar and carbohydrate and lower levels of protein as a percentage of total energy than the standard rodent diets fed to control dams [402-404]. In

addition to the higher total fat content, the fatty acid composition of cafeteria diets used in these studies is also markedly different to the control diets, with higher proportions of saturated and trans fatty acids and a lower n-3 LCPUFA content [159, 189, 195, 401]. This is significant since, of all the components of the maternal diet, the amount of fat and the content of individual fatty acid classes are most closely reflected in their levels in breast-milk [190, 191, 402, 405-407]. However, while previous studies have reported that the total fat content of milk from mothers fed a cafeteria diet is significantly increased relative to those fed on standard diets, there are currently no studies which have determined how these diets affect the fatty acid composition of the milk or the fatty acid status of the offspring.

Thus, the aims of this Chapter were to firstly, determine the impact of providing dams with a cafeteria diet on the fat and protein content and fatty acid composition of their milk, and secondly, to determine the extent to which the fatty acid composition of the dams diet and milk related to the fat mass of the offspring at weaning and fatty acid status of the offspring during the suckling and post-weaning periods. A secondary aim was to determine, using a cross-fostering approach, whether the fatty acid status of the offspring suckled by cafeteria-fed dams differed according to whether or not they were exposed to the cafeteria diet *in utero*.

3.2 MATERIALS AND METHODS

3.2.1 Animals and feeding regime

All procedures were approved by the Adelaide University Animal Ethics Committee. The same procedures were followed as described in Chapter 2.

3.2.2 Measurement of food intake

For both the Control and CAF dams, food intake was determined every two days and fresh food provided. For the Control dams, the weight of feed remaining at the end of the two day period was subtracted from the amount initially provided to determine the weight of feed consumed. For the CAF dams, the weight of each individual food type was subtracted from the amount of that food initially provided to determine the intake of each separate component of the cafeteria diet. The weight of each food consumed was multiplied by the energy, macronutrient or fatty acid composition of the respective food type in order to calculate the intake of total energy, fat, protein, carbohydrate and each of the individual fatty acid classes for each experimental animal. The values obtained for nutritional intake of each dam during pregnancy and lactation were used to provide separate average nutritional intake during the pregnancy and lactation periods for each dam.

3.2.3 Mating and pregnancy

The same procedures were followed as described in Chapter 2.

3.2.4 Cross-fostering

Cross-fostering procedures were conducted as described in Chapter 2. All dams were allowed to give birth naturally. The number of pups in each litter and the sex and birth weight of each pup were measured and all litters culled to 8 pups, with 4 males and 4 females where possible, within 24 hours of birth (culled pups were used for PND1 samples, see below).

3.2.5 Blood sample collection

Blood samples were collected from the Control and CAF offspring within 24 hrs of birth (PND1) and one male and one female pup from each litter at 3 weeks (weaning) and 6 weeks of age. All sampled animals were euthanised with an overdose of CO_2 and blood was collected by cardiac puncture. The blood was centrifuged at 3,500g at 4^0C for 15 minutes. The plasma was removed and the red blood cells (RBCs) prepared for analysis of fatty acid composition as previously described [444].

3.2.6 Milk collection

Milk samples were collected from all dams during the second week of lactation. Dams were separated from their litters for 2-3 hour and were given a single intraperitoneal injection of oxytocin (0.5ml) 5 minutes prior to milking. The milk was expressed from the teats by gentle manual kneading with repetitive top to bottom stroking motions. Between 0.5 to 1.0ml of milk was obtained from each dam and milk samples were frozen at -20^0C until further analysis. Protein concentration of the mid-lactation milk samples was determined by a validated Bradford method using bovine serum albumin as the standard [445].

3.2.7 Determination of total fat content and fatty acid composition

The total lipid concentration of RBCs and milk samples was determined following the protocol of Bligh and Dyer [446] using chloroform-methanol (2:1, v/v). Total lipids were also extracted from a sample of standard feed and each individual component of the cafeteria diet for the assessment of their fatty acid composition. In the RBCs, the phospholipids were separated from total lipid extracts by thin layer chromatography (TLC) on silica gel plates (Silica gel 60H; Merck, Darmstadt, Germany). A lipid class standard 18-5 (NU-CHEK Prep; Elysian, MN) was run on the plates for lipid identification. The mobile phase for

TLC was petroleum spirit/acetone (3:1, v/ v). The TLC plates were sprayed with fluorescein 5-isothiocyanate in methanol, and the lipid classes present were visualised under UV light. The phospholipid bands located at the bottom of TLC plate were transferred into a vial containing 1% sulphuric acid (H_2SO_4) in methanol. All solvents used for extraction and separation contained 0.005% (w/v) antioxidant, butylated hydroxyl toluene (BHT).

All lipids from the milk samples, foods and phospholipids from the RBCs were transesterified with 1% H_2SO_4 in methanol at 70^0C for 3 hours. After the samples were cooled, the resulting fatty acid methyl esters (FAME) were extracted with *n*-heptane and transferred into vials containing a scoop of anhydrous sodium sulphate. FAMEs were separated and quantified by GC (Hewlett-Packard 6890; Palo Alto, CA) equipped with a capillary column (50m x 0.32mm id) coated with 0.25µm film thickness silica (BPX-70; SGC Pty Ltd, Victoria, Australia), and a flame ionisation detector (FID). The injector temperature was set at 250°C and the FID temperature at 300^0C. The oven temperature at injection was initially set at 140°C and was programmed to increase to 220^0C at a rate of 5^0C per minute. Helium gas was utilised as a carrier at a flow rate of 35cm per second in the column. The identification and quantification of FAMEs were achieved by comparing the retention times and peak area % values of unknown samples to those of commercial lipid standards (NU-CHEK Prep; Elysian, MN) using the Hewlett-Packard Chemstation data system. All solvents used in these experiments were of analytical grade and were purchased from Ajax Finechem Pty Ltd (Auckland, New Zealand) or Chem-Supply (South Australia, Australia). Other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

3.2.8 Statistical analysis

Data are presented as mean ± SEM. The effect of maternal diet on the dams' total energy, protein, carbohydrate and fat intake and the intake of each of the individual fatty acid classes during pregnancy and lactation was determined

using a Student's unpaired t-test. Differences in the levels of protein, total fat and individual fatty acids of milk samples and RBC phospholipids on PND1 between the Control and CAF groups were similarly determined. The impact of prenatal and postnatal maternal diet and their interaction on offspring RBC fatty acid composition at 3 and 6 weeks of age was determined separately in male and female pups using a 2 way ANOVA. Where significant interactions were identified by ANOVA, the effect of the prenatal and postnatal maternal diet were analysed separately using a Student's T-test. Relationships between the dam's intake of fat and fatty acids during lactation and the composition of the milk samples were determined using linear regression analysis. Relationships between the fatty acid composition of maternal diet, dam milk samples and RBC phospholipids and fat mass of the offspring were similarly determined. All statistical analyses were conducted using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The litter (mother) was used as the unit of analysis for all statistical tests. A probability of $P<0.05$ was considered statistically significant. Grubb's tests were performed to identify significant outliers in each group prior to the statistical analysis.

3.3 RESULTS

3.3.1 Fatty acid composition of the cafeteria diet

The fatty acid composition of the standard rat feed and each item in the cafeteria diet is shown in Table 3.1. All foods included in the cafeteria diet contained a higher proportion of saturated fats and lower levels of n-3 LCPUFA compared to the standard rodent feed. The content of trans fatty acids was also significantly higher in the lard plus rat feed mix than in any other food item tested (Table 3.1).

3.3.2 Maternal nutritional intake

Mean daily energy intake was about 10% higher in CAF dams during pregnancy and 16% lower during lactation compared to Control dams (Table 3.2). Dams fed the cafeteria diet consumed 4- to 5-fold more fat, but 40% and 54% less protein during pregnancy and lactation respectively compared to Control dams (Table 3.2).

Dams in the CAF group consumed significantly more saturated fats (9-fold), monounsaturated fats (6-fold), trans fatty acids (25-fold), linoleic acid (LA, 18:2n-6), total n-6 PUFA and α-linolenic acid (ALA) (all ~1.5-fold) as a percentage of their total daily energy intake compared to Control dams during both pregnancy and lactation (Table 3.2). The intake of arachidonic acid (AA, 20:4n-6) and the n-3 LCPUFA, eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) as a percentage of total energy intake was, however, significantly lower (by 4- to 30-fold) in CAF dams during pregnancy and lactation compared to Control dams (Table 3.2).

Table 3.1 Fatty acid composition (percent) of the total fat in each diet item

Fatty acids (% total fatty acids)	Control Diet	Cafeteria diet					
	Standard rat chow (5% fat)	Chocolate Biscuits (45% fat)	Savoury Snacks (35% fat)	Peanut butter (50% fat)	Hazelnut spread (36% fat)	Sweetened cereal (3% fat)	Lard & chow (19% fat)
Total saturates	19.30	50.41	46.84	17.81	23.64	38.23	44.71
Total monos	37.05	35.03	41.39	77.29	55.03	35.49	40.96
Totals trans	0.21	0.38	0.65	0.04	0.04	0.09	4.13
LA	36.66	13.34	10.55	4.64	16.50	24.86	7.87
AA	0.07	0.00	0.00	0.00	0.00	0.00	0.02
Total n-6	36.91	13.39	10.55	4.69	16.60	24.96	8.09
ALA	5.78	0.62	0.41	0.09	4.55	0.93	1.60
EPA	0.16	0.00	0.00	0.00	0.00	0.00	0.02
DPA	0.06	0.00	0.00	0.00	0.00	0.00	0.02
DHA	0.45	0.00	0.00	0.00	0.00	0.00	0.10
Total n-3	6.47	0.63	0.41	0.09	4.57	0.93	1.92

Abbreviations: LA, Linoleic acid (18:2 n-6); AA, Arachidonic acid (20:4 n-6); ALA, α-linolenic acid (18:3 n-3); EPA, Eicosapentaenoic acid (20:5 n-3); DPA, Docosapentaenioc acid (22:5 n-3); DHA, Docosahexanoic acid (22:6 n-3).

Table 3.2 Maternal intake of fat (g/day), protein (g/day), total energy (KJ/day) and key fatty acids as a proportion of daily energy intake (%en) during pregnancy and lactation in control and cafeteria fed groups. Values are expressed as means ± SEM. ** denotes significance at *P<0.01* and *** denotes significance at *P<0.001.*

Dietary intake	Pregnancy Period		Lactation Period	
	Control Group	Cafeteria Group	Control Group	Cafeteria Group
Fat (g/day)	1.14 ± 0.03	6.68 ± 0.28 **	2.51 ± 0.06	10.77 ± 0.49 **
Protein (g/day)	4.86 ± 0.12	2.88 ± 0.1 **	10.70 ± 0.26	4.98 ± 0.24 **
Energy (g/day)	446.72 ± 10.76	500.46 ± 12.22 **	982.59 ± 23.54	821.29 ± 27.54 **
Total Saturates (%)	1.91 ± 0.05	17.68 ± 0.79 ***	1.85 ± 0.04	19.03 ± 0.71 ***
Total Monos (%)	3.66 ± 0.09	25.93 ± 1.24 ***	3.55 ± 0.08	26.95 ± 1.30 ***
Totals Trans (%)	0.02 ± 0.0005	0.49 ± 0.04 ***	0.02± 0.0004	0.66 ± 0.03 ***
LA (%)	3.62 ± 0.09	5.46 ± 0.24 ***	3.51 ± 0.08	5.30 ± 0.22***
AA (%)	0.01 ± 0.0002	0.002 ± 0.0002 ***	0.01 ± 0.0001	0.003 ± 0.0002 ***
Total n-6 (%)	3.65 ± 0.09	5.50 ± 0.24 ***	3.53 ± 0.08	5.35 ± 0.23 ***
ALA (%)	0.57 ± 0.01	0.78 ± 0.05 ***	0.55 ± 0.01	0.71 ± 0.03***
EPA (%)	0.02 ± 0.0004	0.002 ± 0.0002 ***	0.01 ± 0.0003	0.0003 ± 0.0002 ***
DPA (%)	0.01 ± 0.0001	0.002 ± 0.0002***	0.01 ± 0.0001	0.0003 ± 0.0002 ***
DHA (%)	0.04 ± 0.001	0.01 ± 0.001 ***	0.04 ± 0.001	0.01 ± 0.001 ***
Total n-3 (%)	0.64 ± 0.02	0.81 ± 0.06 **	0.62 ± 0.01	0.75 ± 0.03***

Abbreviations: %en, Percentage of total daily energy intake; LA, Linoleic acid (18:2 n-6); AA, Arachidonic acid (20:4 n-6); ALA, α-linolenic acid (18:3 n-3); EPA, Eicosapentaenoic acid (20:5 n-3); DPA, Docosapentaenioc acid (22:5 n-3); DHA, Docosahexanoic acid (22:6 n-3).

3.3.3 Milk composition

The milk from dams in the CAF group had a 1.3-fold higher fat content compared to the Controls (Figure 3.1A). There was no difference in the protein content of the milk between groups (Figure 3.1A). The milk from dams consuming the cafeteria diet also contained higher levels of saturated fat (1.3-fold), trans fatty acids (12.2-fold), LA (1.4-fold) and total n-6 PUFA (1.3-fold) as a percentage of total lipids compared to the milk from Control dams (Figure 3.1B). There were no differences in the AA, ALA or n-3 LCPUFA content of the milk between groups (Figure 3.1B).

The total fat content (r^2= 0.58, *P<0.05*) and the levels of saturated (r^2= 0.81, *P<0.01*) and trans fatty acids (r^2= 0.92, *P<0.01*) in the milk were directly related to maternal dietary intake during lactation. There were no relationships, however, between maternal intake of n-6 or n-3 PUFA during the lactation period and the levels of these fatty acids in the milk at mid-lactation.

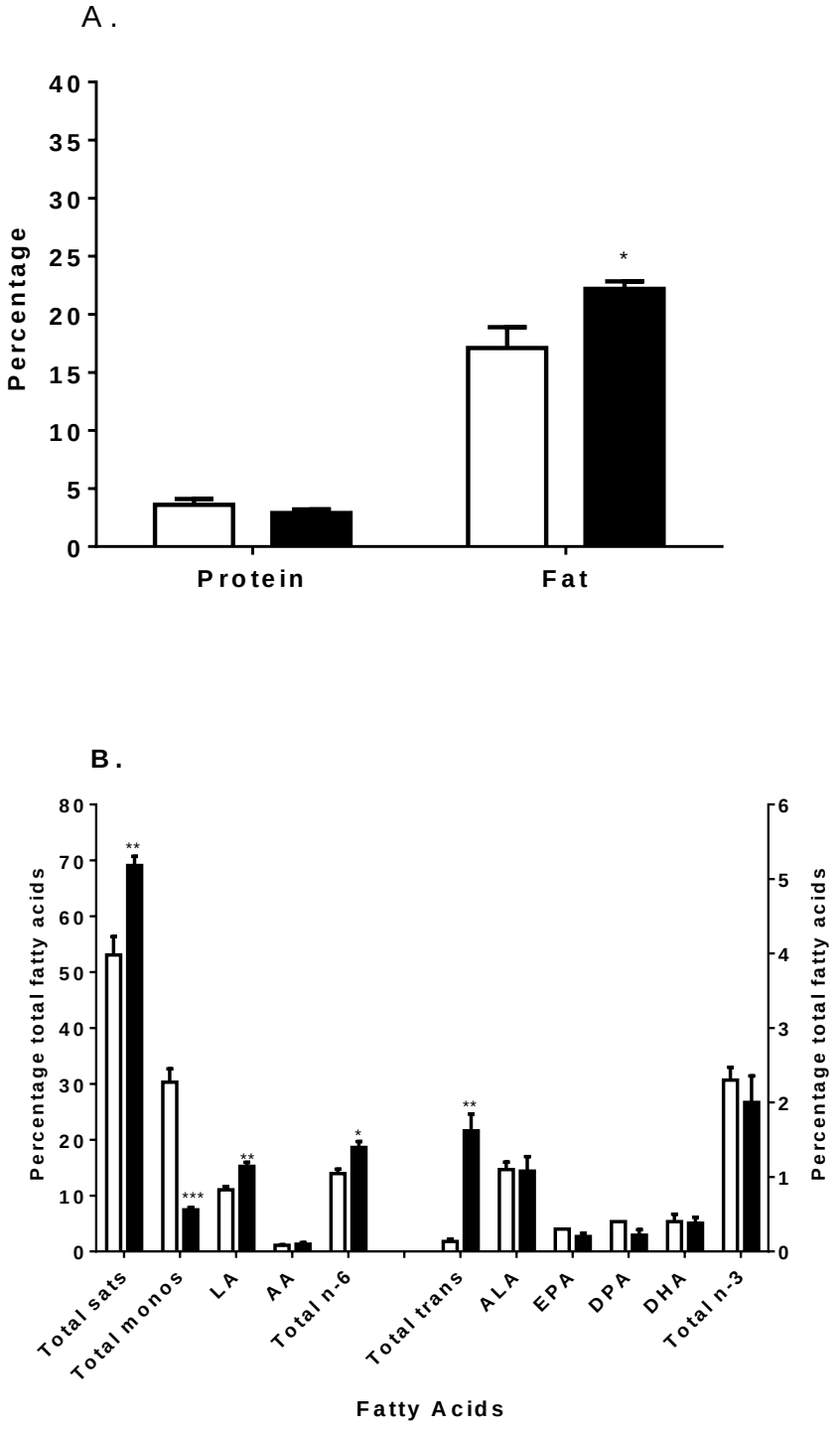


Figure 3.1 The effect of cafeteria feeding on total protein and total fat percentage (A) and fatty acid composition as a percentage of total lipids (B) in the milk of Control dams (open bars) and Cafeteria dams (solid bars). Values are expressed as mean ± SEM. * denotes significance at *P<0.05*, ** denotes significance at *P<0.01* and *** denotes significance at *P<0.001*.

3.3.4 Effect of maternal diet on offspring fatty acid status

3.3.4.1 *Postnatal day 1*

On PND1, pups born to CAF dams had lower LA, EPA, DPA, DHA and total n-3 LCPUFA levels in their RBC phospholipids compared to pups of Control dams (Figure 3.2). Conversely, the level of monounsaturated fats as a percentage of total fatty acids was higher in the CAF group on PND1 (Figure 3.2). There were no differences in the proportions of saturated fat, trans fatty acids, AA or total n-6 PUFA in RBC phospholipids between the CAF and Control pups (Figure 3.2).

The proportions of n-3 LCPUFA in the RBC phospholipids on PND1 were directly related to maternal intake of these fatty acids during pregnancy (EPA, r^2= 0.58, *P<0.01*; DPA, r^2= 0.53, *P<0.05*; DHA, r^2= 0.65, *P<0.01*). There was no relationship, however, between maternal intake of total fat, saturated fat, trans fatty acids, ALA or n-6 PUFA during pregnancy and the level of these respective components in the RBC phospholipids of the offspring on PND1.

3.3.4.2 *3 weeks*

There were no differences in the levels of saturated fat, ALA or AA in RBC phospholipids between groups at 3 weeks of age in either males or females (Table 3.3). At 3 weeks of age, female pups suckled by CAF dams had lower levels of LA, total n-3 LCPUFA and total n-6 PUFA in their RBC phospholipids compared to those suckled by Controls (Table 3.3). In male offspring, however, only LA and DPA content was significantly reduced in the RBC phospholipids of pups suckled by CAF dams (Table 3.3). The levels of trans and monounsaturated fats were higher in both male and female pups suckled by CAF dams compared to those suckled by Controls (Table 3.3). These effects were all independent of whether the pups had been born to a control or CAF dam.

There were no direct relationships between the levels of any of the fatty acid classes in RBC phospholipids of the pups at 3 weeks of age and levels of the respective fatty acids in the maternal milk in either males or females. However, there were direct relationships between the average maternal intake of trans fatty acids and monounsaturated fats during the suckling period and the level of these respective components in the RBC phospholipids of both male and female offspring at 3 weeks of age (male: trans fatty acids, r^2= 0.39, *P<0.01*; monos, r^2= 0.49, *P<0.001*; female: trans fatty acids, r^2= 0.52, *P<0.001*; monos, r^2= 0.70, *P<0.001*). In females, but not in males, levels of AA and the proportions of n-3 LCPUFA in the RBC phospholipids at 3 weeks of age were also directly correlated with maternal intake of these fatty acids during the suckling period (AA, r^2= 0.17, *P<0.05*; EPA, r^2= 0.41, *P<0.001*; DPA, r^2= 0.50, *P<0.001*; DHA, r^2= 0.22, *P<0.05*; total n-3 PUFA, r^2= 0.20, *P<0.05*). There were no significant correlations between the proportions of total saturated fat, total n-6 PUFA or ALA in the RBC phospholipids and maternal intake of these respective fatty acids during the suckling period in either male or female offspring.

3.3.4.3 *6 weeks*

There were no differences in LA, AA, total n-6 PUFA, ALA, EPA and DHA status between groups at 6 weeks in either males or females (Table 3.3). Male pups suckled by CAF dams had lower levels of DPA and total n-3 PUFA and higher levels of trans fatty acids in their RBC phospholipids compared to those suckled by Control dams (Table 3.3). These differences were not present in female offspring (Table 3.3). Both male and female pups suckled by CAF dams had lower levels of saturated fatty acids in the RBC phospholipids at 6 weeks of age compared to those suckled by Controls (Table 3.3). Again, as at 3 weeks, these relationships were independent of whether the pups were born to a Control or CAF dam.

In males, but not in females, the levels of trans fatty acids in RBC phospholipids at 6 weeks of age were directly related to the levels of trans fatty acids in their

foster mother's milk (r^2= 0.73, *P<0.05*). In females, levels of AA in the offspring RBC were directly correlated (r^2= 0.86, *P<0.01*) with the proportions of these fatty acids in the milk.

There were no significant correlations between the proportions of total saturated fat, total n-6 PUFA, ALA, or n-3 LCPUFA in the RBC phospholipids and the level of these respective fatty acids in the mother's milk in either male or female offspring. There were, however, direct relationships between maternal intake of monounsaturated fatty acids during the suckling period and the level of these fatty acids in the RBC phospholipids of both male and female offspring at 6 weeks of age (male: r^2= 0.17, *P<0.05*; female: r^2= 0.37, *P<0.01*). In males, but not in females, levels of trans fatty acids in the RBC phospholipids at 6 weeks of age were also directly correlated with the maternal intake of these fatty acids during the suckling period (r^2= 0.50, *P<0.001*). There were no significant correlations between the proportions of total saturated fat, LA, AA, total n-6 PUFA or the proportions of n-3 LCPUFA in the RBC phospholipids and maternal intake of these respective fatty acids during the suckling period in either male or female offspring at 6 weeks of age.

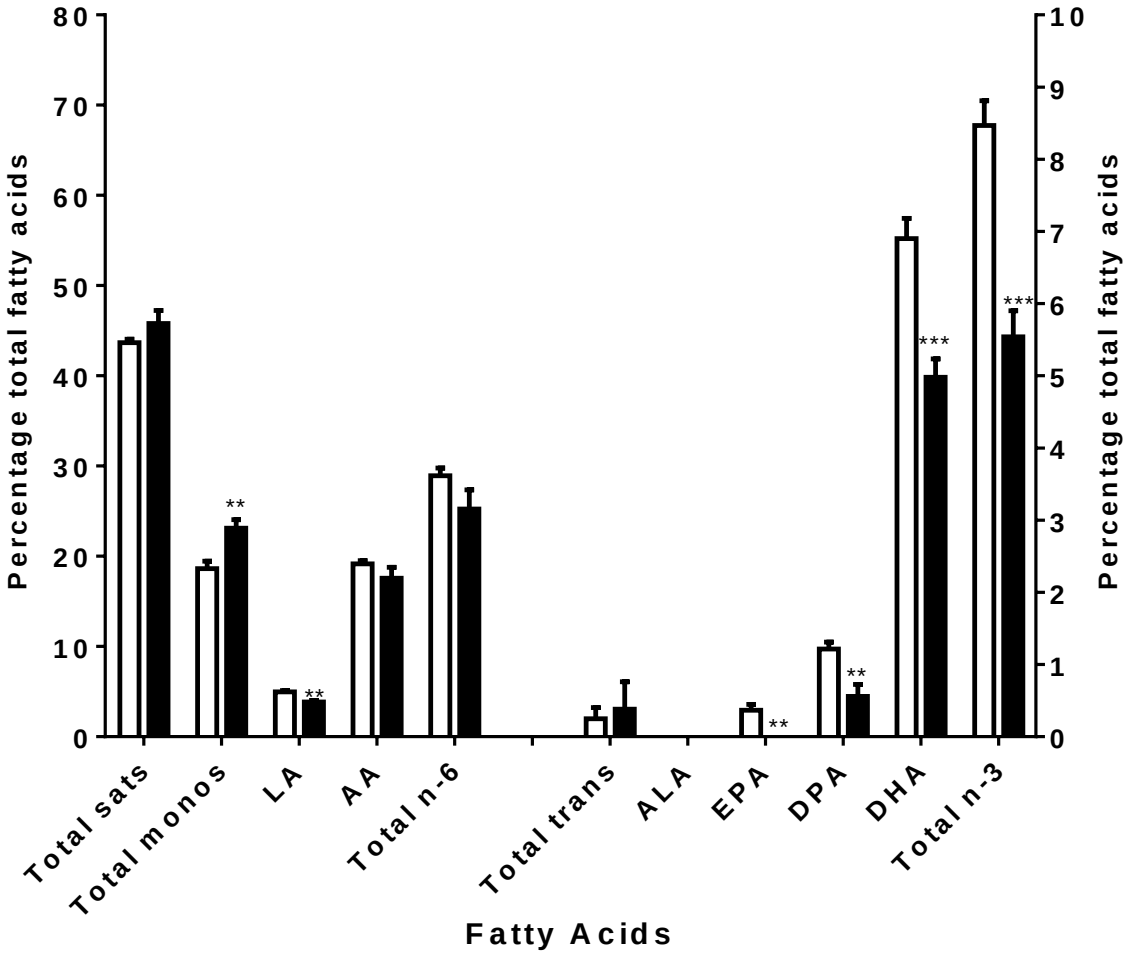


Figure 3.2 The effect of cafeteria feeding on offspring fatty acid status as a percentage of total lipids in the postnatal day 1 red blood cell phospholipids of pups exposed to control diet (open bars) and cafeteria diet (solid bars) during pregnancy. Values are expressed as mean ± SEM. ** denotes significance at *P<0.01* and *** denotes significance at *P<0.001*.

Table 3.3 Red blood cell phospholipid fatty acid composition expressed as a percentage of total fatty acids in male and female offspring of C-C, CAF-C, C-CAF and CAF-CAF groups at 3 weeks and 6 weeks of age. Values are expressed as means ± SE.* denotes significance at *P<0.05*, ** denotes significance at *P<0.01*, and *** denotes significance at *P<0.001*.

3 Weeks

Fatty acids	Male				Female			
%	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Total saturates	49.96 ± 1.4	48.9 ± 3.7	49.80 ± 4.3	44.98 ± 0.81	50.94 ± 1.9	49.97 ± 2.6	55.76 ± 2.42	51.45 ± 2.92
Total monos	11.61 ± 0.53	12.8 ±0.85	16.00 ± 0.31**	15.02 ± 0.96**	11.59 ± 0.45	11.52 ± 0.35	16.64 ± 0.77**	16.16 ± 0.58**
Total trans	0.05 ± 0.03	0.30 ± 0.17	0.78 ± 0.14**	0.51 ± 0.15**	0.05 ± 0.03	0.35 ± 0.12	0.86 ± 0.18**	0.75 ± 0.15**
LA	10.38 ± 0.29	9.45 ± 0.32	8.27 ± 0.61**	9.12 ± 0.30**	9.74 ± 0.16	10.55 ± 0.48	7.96 ± 0.51***	8.59 ± 0.31***
AA	18.36 ± 0.88	17.63 ± 2.29	17.10 ± 2.69	20.30 ± 0.41	18.28 ± 1.05	16.90 ± 1.64	12.92 ± 1.88	15.88 ± 1.96
Total n-6	30.9 ± 1.04	29.23 ± 2.91	27.60 ± 2.8	32.15 ± 0.59	30.25 ± 1.42	29.67 ± 1.38	20.6 ± 3.5**	26.36 ± 2.38**
ALA	0.03 ± 0.02	0.03 ± 0.025	0.00 ± 0.00	0.05 ± 0.02	0.01 ± 0.01	0.05 ± 0.022	0.00 ± 0.00	0.03 ± 0.02
EPA	0.29 ± 0.05	0.30 ± 0.11	0.17 ± 0.03	0.20 ± 0.05	0.30 ± 0.07	0.33 ± 0.05	0.08 ± 0.04**	0.13 ± 0.04**
DPA	2.28 ± 0.23	2.85 ± 0.46	1.43 ± 0.43**	1.85 ± 0.04**	2.36 ± 0.23	2.53 ± 0.39	0.98 ± 0.20***	1.21 ± 0.25***
DHA	4.85 ± 0.59	5.50 ± 0.83	3.93 ±1.08	5.13 ± 0.23	4.46 ± 0.59	5.45 ± 0.52	2.78 ± 0.70*	3.74 ± 0.56*
Total n-3	7.44 ± 0.84	8.70 ± 1.37	5.60 ± 1.52	7.22 ± 0.22	7.13 ± 0.82	8.40 ± 0.94	3.86 ± 0.92**	5.10 ± 0.82**

6 Weeks

Fatty acids	Male				Female			
%	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Total saturates	47.99 ± 0.27	47.75 ± 0.72	47.05 ± 0.46*	46.86 ± 0.33*	48.16 ± 0.39	50.97 ± 1.72	48.07± 0.41	45.97± 1.31
Total monos	13.06 ± 0.32	13.88 ± 0.27	14.48 ± 0.23*	13.97 ± 0.26*	12.55 ± 0.26	12.63 ± 0.35	14.02 ± 0.15***	13.99 ± 0.44***
Total trans	0.04 ± 0.01	0.09 ± 0.03	0.15 ± 0.03**	0.15 ± 0.02**	0.029 ± 0.02	0.15 ± 0.06	0.07 ± 0.03	0.06 ± 0.03
LA	9.78 ± 0.21	9.93 ± 0.19	9.63 ± 0.21	9.90 ± 0.24	9.11 ± 0.33	8.92 ± 0.34	8.83 ± 0.14	8.90 ± 0.21
AA	19.79 ± 0.42	18.68 ± 0.94	19.28 ± 0.56	19.80 ± 0.43	21.28 ± 0.35	19.03 ± 0.99	20.10 ± 0.42	22.00 ± 0.68
Total n-6	32.23 ± 0.38	31.08 ± 0.83	31.73 ± 0.48	32.54 ± 0.05	32.83 ± 0.47	30.13 ± 0.91	31.57 ± 0.42	33.44 ± 0.83
ALA	0.09 ± 0.01	0.08 ±0.03	0.10 ± 0.00	0.10 ± 0.00	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.09 ± 0.01
EPA	0.32 ± 0.02	0.43 ± 0.03	0.38 ± 0.025	0.34 ± 0.02	0.36 ± 0.02	0.38 ± 0.03	0.35 ± 0.02	0.37 ± 0.03
DPA	2.40 ± 0.05	2.45 ± 0.05	2.20 ± 0.071***	2.10 ± 0.04***	2.13 ± 0.09	1.97 ± 0.22	1.90 ± 0.09	1.97 ± 0.12
DHA	3.80 ± 0.06	4.20 ± 0.21	3.95 ± 0.05	3.79 ± 0.06	3.73 ± 0.13	3.60 ± 0.44	3.90 ± 0.19	4.01 ± 0.20
Total n-3	6.64 ± 0.06	7.18 ± 0.19	6.68 ± 0.06	6.37 ± 0.08**	6.36 ± 0.21	6.02 ± 0.68	6.23 ± 0.27	6.49 ± 0.33

Abbreviations: %, Percentage of total fatty acids; LA, Linoleic acid (18:2 n-6); AA, Arachidonic acid (20:4 n-6); ALA, α-linolenic acid (18:3 n-3); EPA, Eicosapentaenoic acid (20:5 n-3); DPA, Docosapentaenioc acid(22:5 n-3); DHA, Docosahexanoic acid (22:6 n-3).

3.3.5 The relationship between maternal diet, milk composition and fat mass in the male and female offspring at weaning

Total relative fat mass of the offspring at weaning was directly related to the percentage of saturated, monounsaturated fats and trans fatty acids in the maternal milk in both males and females (Table 3.4). In males, but not in females, total fat content and the levels of LA and total n-6 PUFA in the maternal milk were also directly correlated with total relative fat mass at weaning (Table 3.4). There were no significant correlations between the proportions of AA and the proportions of n-3 LCPUFA in the maternal milk and total fat mass in either male or female offspring at weaning (Table 3.4).

At weaning, maternal intake of total fat, saturated fat, total monos, trans fatty acids and the proportions of both n-6 and n-3 PUFA were each directly related to relative total fat mass in both male and female offspring (Table 3.4).

Table 3.4 The relationship between maternal total fat (%en) and fatty acid intake (%en), total fat (%) and fatty acid composition (%) in the milk and total fat mass relative to body weight in the male and female offspring at weaning.

Components	Maternal Diet		Milk composition	
	Male	Female	Male	Female
Total fat	r^2 = 0.90, *P< 0.001*	r^2 = 0.78, *P< 0.001*	r^2 = 0.57, *P< 0.05*	NS
Total saturates	r^2 = 0.90, *P< 0.001*	r^2 = 0.80, *P< 0.001*	r^2 = 0.71, *P< 0.05*	r^2 = 0.96, *P< 0.01*
Total monos	r^2 = 0.87, *P< 0.001*	r^2 = 0.77, *P< 0.001*	r^2 = 0.90, *P< 0.01*	r^2 = 0.91, *P< 0.01*
Total trans	r^2 = 0.87, *P< 0.001*	r^2 = 0.80, *P< 0.001*	r^2 = 0.85, *P< 0.01*	r^2 = 0.74, *P< 0.05*
LA	r^2 = 0.80, *P< 0.001*	r^2 = 0.59, *P< 0.001*	r^2 = 0.80, *P< 0.01*	NS
AA	r^2 = 0.76, *P< 0.001*	r^2 = 0.67, *P< 0.001*	NS	NS
Total n-6	r^2 = 0.80, *P< 0.001*	r^2 = 0.60, *P< 0.001*	r^2 = 0.80, *P< 0.01*	NS
ALA	r^2 = 0.67, *P< 0.001*	r^2 = 0.39, *P< 0.01*	NS	NS
EPA	r^2 = 0.85, *P< 0.001*	r^2 = 0.77, *P< 0.001*	NS	NS
DPA	r^2 = 0.70, *P< 0.001*	r^2 = 0.61, *P< 0.001*	NS	NS
DHA	r^2 = 0.84, *P< 0.001*	r^2 = 0.75, *P< 0.001*	NS	NS
Total n-3	r^2 = 0.59, *P< 0.001*	r^2 = 0.300, *P< 0.01*	NS	NS

Abbreviations: NS, No significant correlations; LA, Linoleic acid (18:2 n-6); AA, Arachidonic acid (20:4 n-6); ALA, α-linolenic acid (18:3 n-3); EPA, Eicosapentaenoic acid (20:5 n-3); DPA, Docosapentaenioc acid (22:5 n-3); DHA, Docosahexanoic acid (22:6 n-3).

3.4 DISCUSSION

The results of this Chapter have demonstrated that the relationship between maternal fatty acid intake, milk composition, and fatty acid status of the offspring differed markedly between individual fatty acid classes and offspring age. A key finding of this study, however, was that, for both total fat content and all individual fatty acid types, the differences in fat content and fatty acid composition of the milk and offspring RBC phospholipids between groups were much smaller than differences in their levels in the maternal diet, suggesting that the dam has a substantial capacity to buffer the transfer of fats from the diet into the milk supply. Despite this, however, the present study identified positive relationships between both maternal intake and milk content of specific fatty acid classes and the relative fat mass of the offspring at 3 weeks of age, suggesting that these may be important drivers of the increased fat mass of offspring suckled by CAF dams reported in Chapter 2.

3.4.1 Maternal diet and milk composition

Consistent with previous studies, analysis of the cafeteria diet revealed high levels of saturated and trans fatty acids and lower levels of n-3 LCPUFA in the majority of the food items in comparison to the standard diet [139, 140, 164, 447]. This translated into significantly higher intakes of saturated and trans fatty acids and lower intakes of n-3 LCPUFA in the cafeteria fed dams compared to those consuming the standard diet. Dams consuming the cafeteria diet also had a higher n-6 PUFA intake, resulting in a significantly higher dietary n-6 to n-3 PUFA ratio in this group. This pattern of dietary fat intake, i.e. high levels of saturates, trans fatty acids and particularly the increased ratio of n-6 to n-3 PUFA is comparable to poor quality western-style diets in humans [448, 449].

Importantly, I demonstrated that the higher maternal intakes of total fat, saturates, trans fatty acids and n-6 PUFA were reflected by higher levels of these components in the dam’s milk, and that levels increased in direct

proportion to maternal intake. These results confirm previous reports of a dose-dependent relationship between total fat content [147, 148] and trans fatty acid levels [203, 450, 451] in the maternal diet and levels in the milk, and suggests that the same is true of saturated fat. The higher n-6 PUFA content in the milk of cafeteria dams is also in line with human studies reporting a positive relationship between maternal n-6 PUFA intakes and increased concentrations of n-6 PUFA in the breast-milk [139, 140]. Importantly, however, the differences in the levels of these components in the milk between control and cafeteria-fed dams were much smaller in magnitude than differences in the level of intake in the maternal diet, indicative of maternal buffering of dietary fat intake.

An unexpected finding was that there were no differences in the n-3 LCPUFA content of the milk samples between control and cafeteria dams, despite the significantly lower level of n-3 LCPUFA intake in the dams consuming the cafeteria diet. A number of human and animal studies have reported a direct relationship between maternal intake of n-3 LCPUFA and their concentrations in the breast-milk [452-454]. However, these studies have largely focused on the impact of n-3 LCPUFA supplementation, rather than reductions in n-3 LCPUFA intake, and it is therefore possible that there are physiological mechanisms to maintain n-3 LCPUFA levels in mature milk when dietary intakes are low. It is also possible that the lower intakes of n-3 LCPUFA in the cafeteria dams were offset by their higher intake of ALA, since rats, unlike humans, have a relatively high capacity for converting ALA through to EPA, DPA and DHA [455].

In contrast to fat, there were no differences in the protein content of the milk between control and cafeteria dams, despite the lower protein intake of the cafeteria dams during both pregnancy and lactation. This is consistent with previous studies in both humans and animals [427, 431, 456, 457] and suggests that lower protein intakes in the maternal diet, at least to the extent observed in the present study, does not translate into a reduced protein supply to the offspring.

3.4.2 Maternal cafeteria diets and offspring fatty acid status

On PND1, the main difference between groups was a lower n-3 LCPUFA status in pups born to cafeteria-fed dams. Since these pups would have commenced milk feeding less than 24 hours before blood collection, their fatty acid status would be expected to be a reflection of *in utero* supply. Thus, these data suggest that the lower n-3 LCPUFA intake of the cafeteria-fed dams resulted in a reduced supply of n-3 LCPUFA to the developing fetus [458-460].

The cross-fostering approach in this study allowed us to dissect out the relative contribution of maternal diet during the fetal and suckling periods to fatty acid status of the offspring at weaning, and the results demonstrated that any differences in fatty acid status in the offspring were related to the diet of their mother during lactation, independent of whether they were born to a control or cafeteria-fed dam.

I also found, however, that the differences observed between offspring suckled by control or cafeteria-fed dams were not always consistent with maternal diet or milk composition. Indeed, offspring suckled by cafeteria-fed dams, particularly females, had a lower content of both n-3 and n-6 PUFA in RBC phospholipids at weaning, in spite of the fact that n-6 PUFA levels were higher and n-3 LCPUFA levels not different in the milk of cafeteria dams. It therefore appears that the n-3 LCPUFA supply during lactation was not sufficient to fully compensate for the lower n-3 LCPUFA status at birth. Interestingly, by 6 weeks of age, the n-3 LCPUFA status of male offspring suckled by a cafeteria dam was still lower than controls. This is unexpected given that all offspring had been consuming the same diet since weaning, and suggests that the fatty acid status at weaning has prolonged effect on n-3 LCPUFA status of the offspring and/or their capacity for incorporation/metabolism of fatty acids derived from the diet. Since the diet provided to the offspring post-weaning contained relatively low amounts of pre-formed n-3 LCPUFA, the majority of these fats would have been derived from ALA. It is well described that females have a higher capacity for ALA conversion than males [461], and this may explain why the female

offspring were able to restore n-3 LCPUFA status to control levels in the 3 weeks after weaning, while males were not.

The increased levels of trans fatty acids in the RBC phospholipids at weaning provides further evidence that trans fatty acids in the maternal diet are transferred to the suckling offspring via the breast-milk, and have persistent effects on the level of trans fatty acids in the offspring. Whether this higher trans fatty acid level has negative physiological consequences for the offspring remains to be determined, but is concerning given the well-described negative impacts of elevated trans fatty acid intake on cardiovascular and metabolic health [462, 463].

3.4.3 Maternal dietary fatty acid intake, milk composition and offspring adiposity at weaning

In order to determine the contribution of changes in maternal milk composition to the increased adiposity in offspring suckled by cafeteria dams at weaning, I assessed relationships between maternal milk and diet composition and fat mass in offspring at 3 weeks of age. Whilst previous studies have reported conflicting results regarding the relationship between maternal dietary fat intake and adiposity in infants and children [208-219], and other studies have failed to find any association between fat intake during infancy and later adiposity [220-222], the results from the present study provide clear evidence that not only the maternal fat ingestion but also the qualitative composition of fatty acids in the maternal diet during lactation play an important role in determining offspring adiposity. Specifically, the data from this Chapter strongly implicates the levels of saturated and trans fatty acids in the milk as key drivers of fat deposition in the offspring during the suckling period.

Whilst, previous studies have reported that the phospholipid fatty acid composition of plasma and RBC is a good reflection of majority of organ tissues in rats [464], previous studies have also suggested that the n-6 and n-3 PUFA

may play an important role in the early development of adipose tissue. This is due to previous findings that n-6 PUFA have pro-adipogenic and pro-lipogenic actions, while n-3 LCPUFA have the opposite effect [182]. While maternal LA intake has not been found to be related to relative adiposity in the offspring in all studies [206, 224], others have reported that increased consumption of n-6 PUFAs, in association with a high n-6/n-3 ratio, has the capacity to promote adipose tissue deposition during the fetal/suckling period and during infancy [139, 223]. In the present study, I found a positive relationship between both LA and total n-6 PUFA content of the milk and relative fat mass at weaning in male, but not female offspring. This suggests, therefore, that the effect of n-6 PUFA on adipogenesis/lipogenesis in the fetal/early postnatal period may be sex-specific, and this will be an important area for future research. The results from the present study are, however, consistent with previous studies which have suggested that the increasing intake of n-6 compared to n-3 PUFA in modern Western diets, reflected in the breast-milk fatty acid composition and n-6/n-3 PUFA ratio, might be an important factor contributing to the current obesity epidemic [139-141, 204-207].

The results of the present study also showed that exposure to higher amounts of trans fatty acids during the perinatal period was positively associated with relative fat mass at weaning in both male and female offspring. Similar associations between higher maternal intake of trans fatty acids during gestation and lactation and increased adiposity in the offspring has been reported in previous studies [225, 465]. These studies also hypothesised that the programming of obesity resulting from early exposure to trans fats may have deleterious consequences and may occur even after trans fat intake ceases in later life [225, 465]. Therefore, dietary trans fatty acids appear to play an important role in driving fat deposition in the offspring in early life, and their role in metabolic programming warrants further investigation. Overall, therefore, the results of this study have provided evidence that the changes in dietary fat intake and fatty acid composition and the associated changes in the total fat and fatty acid composition of maternal milk are likely to play a central role in the increased adiposity in the offspring suckled by CAF dams.

3.5 SUMMARY

The results of this Chapter show that the fatty acid composition of the maternal milk translates into persistent effects on the fatty acid composition of the offspring, particularly for the n-3 LCPUFA and trans fatty acids. However, the maternal dietary intake of fats are not fully reflected in the milk composition, the magnitude of changes being much smaller than the differences in the maternal diet – implying that the dam was buffering the transfer of dietary lipids into the milk. Despite this, however, I found that the levels of specific fatty acids, in particular saturated, trans and n-6 fats, in the maternal milk, were positively related to relative fat mass in the offspring at weaning, providing evidence that the fatty acid composition of the milk supply during the suckling period is an important determinant of fat deposition during this period, and may well account for the higher adiposity at weaning in offspring suckled by CAF dams. Overall, the results from the present study clearly suggest that mothers consuming poor-quality diets, high in trans, n-6 PUFA and saturated fats, and lower in n-3 LCPUFA, are likely to be exposing their offspring lower n-3 LCPUFA levels and elevated levels of n-6 PUFA, saturated and trans fatty acids during critical windows of development. This may be of particular significance given the suggested link between elevated exposure to saturated fats and n-6 PUFA in early life and later risk of obesity, metabolic and allergic diseases, and the potential negative health impacts of exposure to an increased supply of trans fatty acids in early life.

CHAPTER 4

CHAPTER 4: EFFECT OF EXPOSURE TO MATERNAL CAFETERIA DIET DURING THE FETAL AND/OR SUCKLING PERIOD ON ADIPOCYTE GENE EXPRESSION IN THE OFFSPRING

4.1 INTRODUCTION

As highlighted in the literature review, the capacity of pre-adipocytes in humans to proliferate and differentiate during adult life is much lower than before birth and in early infancy and most, if not all, adipose tissue development is completed by the end of the first year of life [235, 257]. Thus, the fetal and early postnatal period are critical windows in the development of adipose depots, and this process is highly sensitive to the nutritional environment an individual experiences during this time, in particular the prevailing concentrations of glucose, insulin and leptin [235, 257].

Studies in sheep, which have a similar profile of adipose cell development to humans, have reported that exposure to excess fetal glucose concentrations in late gestation results in increased expression of the adipogenic and lipogenic genes within adipose cells, including the key adipogenic/lipogenic transcription factor PPAR-γ [232, 257, 375]. Importantly, it has been demonstrated that these changes in adipocyte gene expression persist after birth and are associated with an increased body fat mass in the lamb at the end of the first month of life [375]. Studies in rodents have also demonstrated that the offspring of dams fed a junk-food diet during pregnancy and lactation exhibit increased glucose and lipid intake as well as increased adipocyte proliferation and differentiation in adulthood [91, 117, 400]. In addition, there is evidence from studies in both humans and animal models that relatively minor perturbations in fetal/infant adipose tissue growth and endocrine sensitivity may have important long-term effects for adipose tissue mass [91, 117, 235, 257, 376, 400].

There is considerable evidence that C/EBP-α, and PPAR-γ serve as pleiotropic transcriptional activators that co-ordinately induce expression of a suite of adipocyte specific genes, resulting in the differentiation of adipose cells [466, 467]. PPAR-γ also regulates adipose tissue mass and the expression of genes involved in signalling of adipose cells to other peripheral tissues. Specifically, PPAR-γ decreases the expression of leptin and increases the expression of the insulin sensitising hormone, adiponectin, within adipose tissue [289]. PPAR-γ also upregulates the expression of genes involved in increasing storage of triglycerides within adipose cells, including LPL, FAS and G3PDH [205, 275, 468, 469]. It has been also demonstrated that leptin, a well-characterised regulator of appetite and energy balance, inhibits food intake and weight gain [330], decreases the mass of white adipose tissue [331, 332], promotes lipolysis and reduces lipogenesis in white adipocytes and increases thermogenesis in brown adipocytes [333]. Plasma concentrations of adiponectin are also reported to be positively correlated with neonatal adiposity and adiponectin concentrations are significantly higher in human neonates than in adults [369], suggesting that adiponectin may have a role in promoting growth and fat deposition in early postnatal life.

Although previous studies have demonstrated that exposure to increased maternal nutrition during critical windows of development plays a role in the development of obesity, and that this may be the result of alterations in the biology of adipocyte, the relative importance of exposure to an increased nutrient supply before birth and during early postnatal period for the programming of altered expression of adipocyte regulatory genes have not yet been characterised. It is also not known whether such changes may contribute to the later development of an obese phenotype even if the offspring are weaned onto a control diet.

In Chapter 2 of this thesis, I showed that fat mass at weaning was significantly increased in those pups exposed to a maternal cafeteria diet during the suckling period, irrespective of whether their mother had consumed a control or

cafeteria diet during pregnancy; however the biological mechanisms driving this increased fat mass are unknown. The overall aim of this Chapter was to determine whether the increased fat mass in offspring exposed to a cafeteria diet during the suckling period were due to altered expression of genes regulating adipogenesis and lipogenesis in the subcutaneous and/or retroperitoneal fat pads, and whether these effects were sex-specific. This study also aimed to determine whether any changes in adipogenic, lipogenic and adipokine gene expression in the adipose tissue of these offspring persisted after the pups have been fed on a standard chow diet for 3 weeks after weaning.

4.2 MATERIALS AND METHODS

4.2.1 Animals and feeding regime

All procedures were approved by the Adelaide University Animal Ethics Committee. The same procedures were followed as described in Chapter 2.

4.2.2 Mating and pregnancy

The same procedures were followed as described in Chapter 2.

4.2.3 Cross-fostering

Cross-fostering procedures were conducted as described in Chapter 2.

4.2.4 Determination of plasma glucose, NEFA, insulin and leptin concentrations

Plasma glucose, NEFA, insulin and leptin concentrations were determined as described in Chapter 2. The intra- and inter-assay coefficients of variation (CoV) for glucose and NEFA assays were < 5% and the intra- and inter-assay coefficients of variation (CoV) for insulin and leptin assays were < 10%.

4.2.5 Post-mortem and tissue collection

Post-mortems were carried out as described in Chapter 2. The same procedures for euthanasia and collection and storage of tissues were used as previously described in Chapter 2. Samples of retroperitoneal (visceral fat) and subcutaneous fat were snap frozen in liquid nitrogen and then stored at -80^{0}C for subsequent molecular analysis. The number of pups in each group from whom samples were collected is shown in Table 4.1.

Table 4.1 Total number of animals included in each group at 3 weeks and 6 weeks in males and females.

3 weeks				
Subcutaneous adipose tissue	C-C	CAF-C	C-CAF	CAF-CAF
Male	7	4	4	7
Female	7	5	6	7
Retroperitoneal adipose tissue				
Male	7	4	4	7
Female	8	5	4	7
6 weeks				
Subcutaneous adipose tissue	C-C	CAF-C	C-CAF	CAF-CAF
Male	8	4	4	6
Female	8	5	6	6
Retroperitoneal adipose tissue				
Male	8	4	4	6
Female	8	5	6	6

4.2.5 RNA extraction and reverse transcription

Total RNA was extracted from retroperitoneal and subcutaneous adipose tissue (~100mg) using Trizol reagent (Sigma-Aldrich Co.,St.Louis, USA) and purified using an RNeasy Mini Kit (Qiagen Pty Ltd, Doncaster, Australia). RNA concentration and purity was determined by measuring the absorbance at 260nm and 280nm using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, DE, USA), and RNA integrity was confirmed using agarose gel electrophoresis. ~1µg RNA was then reverse transcribed to generate cDNA using Superscript III reverse transcriptase (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and random hexamers.

4.2.6 Determination of gene expression in the subcutaneous and retroperitoneal adipose tissue

The relative expression of SREBP-1c, PPAR-γ, FAS, G3PDH, leptin and adiponectin, mRNA transcripts was determined by Quantitative real time PCR (qRT PCR) using the SYBR green system on the Applied Biosystems ViiA 7 Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA). All primers had been validated previously for use in rat tissues [205]. All primers were sequenced prior to the experiment to ensure the authenticity of the DNA product and a qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Primer sequences are shown in Table 4.2. Four reference genes (β-actin, GAPDH, HPRT and cyclophilin A (CYPα)) were assessed using the Best keeper program (BestKeeper© Software, Excel© spreadsheet tool using a Repeated Pair-wise Correlation and Regression Analysis, Forum Life Science [470]) to determine which were most stable reference genes across different treatments and fat depots for subsequent experiments. Based on these results, GAPDH and HPRT were deemed unsuitable due to low stability and β-actin and CYPα were used as the reference genes in all the experiments. mRNA expression of the reference gene β-actin was measured using the β-actin Quantitect primer assay (Qiagen Australia,

Doncaster, Vic, Australia). The primer sequences for CYPα are shown in Table 4.2.

For the qRT-PCR measurements, the primer concentrations were consistent for all genes and the amplification efficiency of all primers was 0.997 – 0.999. A constant amount of cDNA (1µl) was used for each qRT-PCR measurement and at least three technical replicates were performed for each gene. Each qRT-PCR reaction well (10µl total volume) contained: 5 µl iTaq™ SYBR® Green supermix 2x (Bio-Rad Laboratories, Hercules, CA); 1 µl of Forward and Reverse primer giving a final concentration of 600 or 900nM, 2µl of molecular grade H_20 and 1.0µl of a 50ng/µl dilution of the stock template. The cycling conditions consisted of 40 cycles of 95^0C for 15 min and 60^0C for 1 min. At the end of each run dissociation melt curves were obtained. Three quality controls as well as two negative controls for each primer were included on each 96-well plate in order to verify inter-plate consistency, and the inter-plate CoV was <5% for all experiments. The abundance of each mRNA transcript was quantified relative to the two housekeeper genes (β-actin and CYPα) using the Applied Biosystems Data Assist software (Applied biosystems, Foster City, CA, USA). This software allows expression of each target gene to be measured against the mean normalised expression of the two housekeepers.

Table 4.2 Primers sequences used for the determination of gene expression in adipose tissue by qRT-PCR.

GENE	Forward Primer (5'-3')	Reverse Primer (5'-3')	Accession No.
SREBP-1c	TGCGGACGCAGTCTGGGCAAC	GTCACTGTCTTGGTTGTTGATG	AF 286469
PPAR-γ	TCCTCCTGTTGACCCAGAGCAT	AGCTGATTCCGAAGTTGGTGG	NM 013124
G3PDH	GCTTCGGTGACAACACCA	AGCTGCTCAATGGACTTTCC	NM 022215
FAS	TGCTCCCAGCTGCAGGC	GCCCGGTAGCTCTGGGTGTA	NM 017332
ADIPONECTIN	AATCCTGCCCAGTCATGAAG	CATCTCCTGGGTCACCCTTA	NM 144744
LEPTIN	ATTTCACACACGCAGTCGGTATCCG	CCAGCAGATGGAGGAGGTC	NM 013076
CYPα	TATCTGCACTGCCAAGACTGAGTG	CTTCTTGCTGGTCTTGCCATTCC	NM 017101

4.2.7 Statistical analyses

Data are presented as mean ± SEM. The effect of maternal diet and sex on offspring plasma hormone and metabolite concentrations and gene expression in the retroperitoneal and subcutaneous tissues of offspring at 3 weeks and 6 weeks were determined using three-way ANOVA, with sex, prenatal and postnatal diet as factors. As three-way ANOVA revealed many differences in mean values and variability of measures between sexes, the effect of prenatal vs postnatal environment and their interaction on offspring plasma hormone and metabolite concentrations and gene expression in the subcutaneous and retroperitoneal tissues were determined separately in male and female offspring using a 2 way ANOVA. Where significant interactions were identified by ANOVA, the effect of the prenatal and postnatal exposure were analysed separately by Student's T-Test.

Relationships between gene expression in subcutaneous and retroperitoneal fat and fat mass of their respective depot in the male and female offspring at 3 weeks and 6 weeks were determined by simple linear regression analysis. Relationships between gene expressions in subcutaneous and retroperitoneal fat and plasma hormone and metabolite concentrations in the male and female offspring at 3 weeks and 6 weeks were similarly determined. Differences in the expression of adipogenic and lipogenic genes between the subcutaneous and retropeitoneal fat in the male and female offspring at 3 weeks and 6 weeks were determined by Student's T-Test. The repeated measure ANOVAs were performed using Stata 10 (Stata Corp LP, Texas, USA). All other analyses were performed using SPSS for Windows Version 17.0 (SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered statistically significant in all analyses. Grubb's tests were performed to identify significant outliers in each group prior to the statistical analysis.

4.3 RESULTS

4.3.1 Expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal adipose tissue at 3 weeks of age

4.3.1.1 *SREBP-1c mRNA expression*

At 3 weeks of age, the relative expression of SREBP-1c in subcutaneous adipose tissue was lower in offspring suckled by CAF dams compared to the controls in males, but was not different in females (Figure 4.1 A, B). In the retroperitoneal depot, however, mRNA expression of SREBP-1c tended (*P=0.057*) to be lower in offspring suckled by a CAF dam compared to the controls in female offspring (Figure 4.1D), but was not different in males (Figure 4.1C).

When data from all groups were combined there was a significant inverse correlation between the expression of SREBP-1c mRNA in the subcutaneous adipose tissue and the relative mass of the subcutaneous fat depot in both male and female offspring (Figure 4.2 A, B). There was also a significant inverse relationship between the expression of SREBP-1c mRNA and relative mass of the retroperitoneal fat depot in female offspring and a trend (r^2= -0.206, *P=0.051*) towards a negative relationship in males (Figure 4.2 C, D).

The expression of SREBP-1c mRNA in the subcutaneous adipose tissue and retroperitoneal adipose tissue was not related to plasma concentrations of glucose, NEFA or leptin in either males or females at 3 weeks of age (Table 4.3). There was however, a negative correlation between the expression of SREBP-1c mRNA in the subcutaneous adipose tissue and plasma insulin concentrations in the male offspring at this time point (Table 4.3). There was no relationship between the expression of SREBP-1c mRNA in the retroperitoneal adipose tissue at 3 weeks of age and plasma concentrations of insulin in either males or in females (Table 4.3).

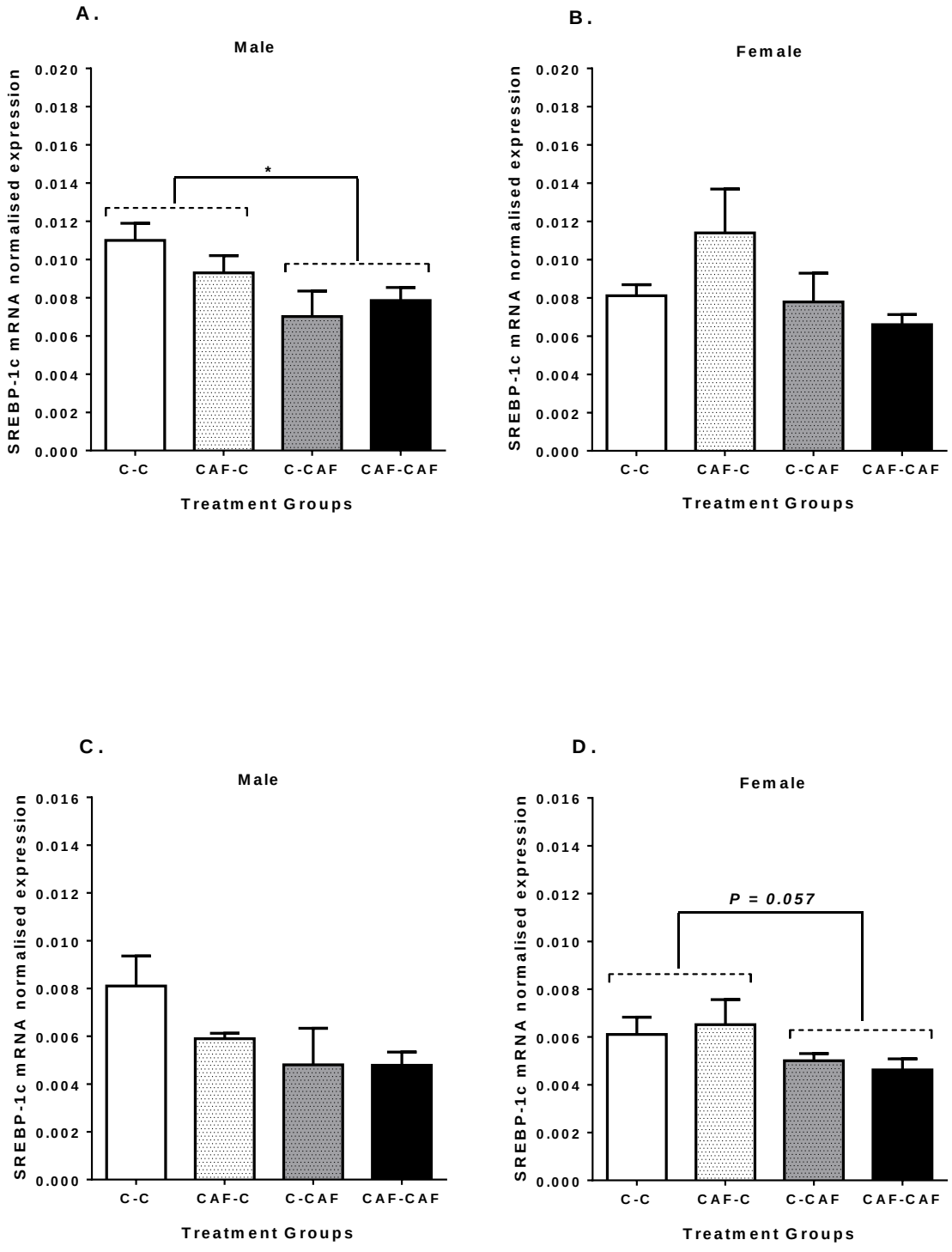


Figure 4.1 The relative expression of SREBP-1c mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

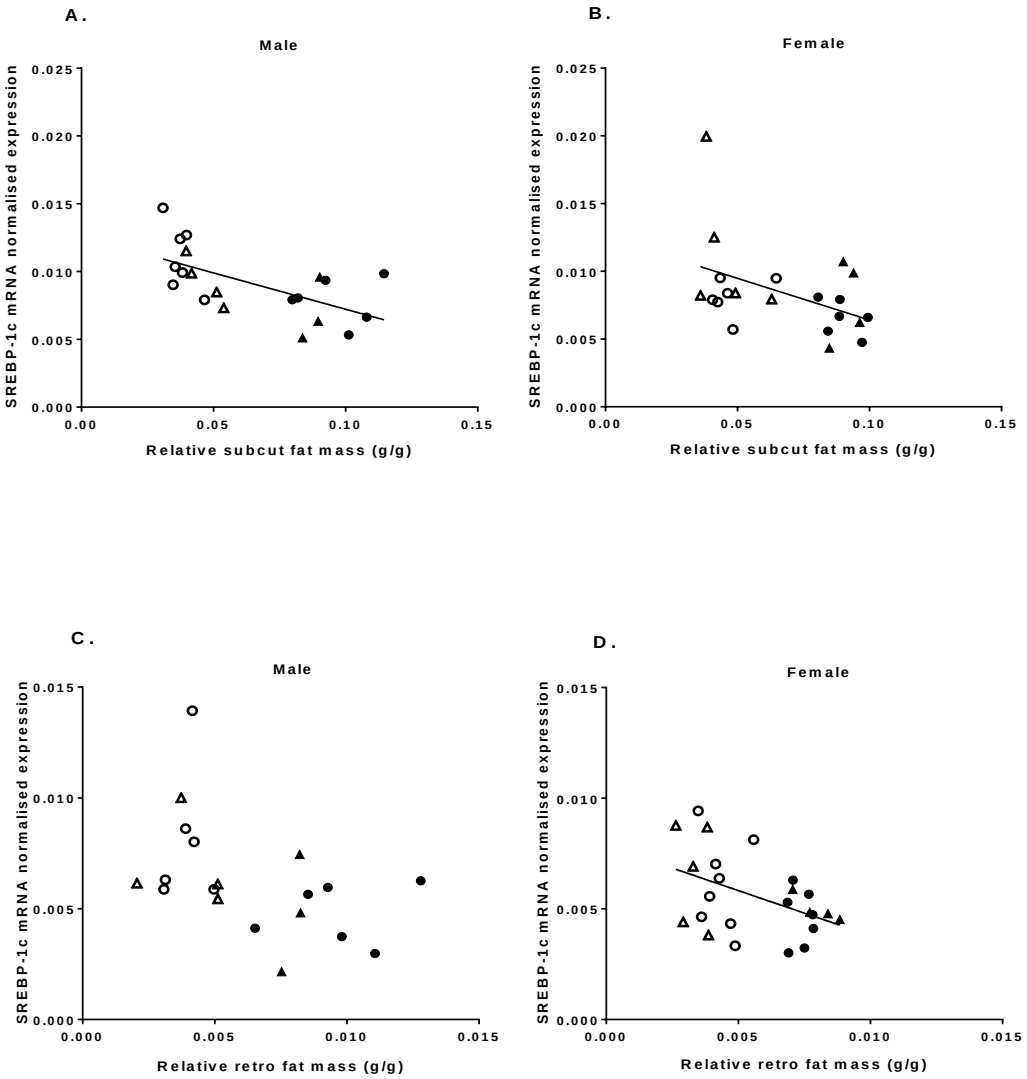


Figure 4.2 The relationship between SREBP-1c mRNA expression in subcutaneous adipose tissue and fat mass in subcutaneous fat depot in the male (A) and female (B) offspring and the relationship between SREBP-1c expression in retroperitoneal adipose tissue and fat mass in retroperitoneal fat depot in the male (C) and female (D) offspring at 3 weeks of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant negative relationship between the expression of SREBP-1c mRNA in the subcutaneous adipose tissue and fat mass in the subcutaneous fat depot in both male (A: r^2= -0.380, *P<0.01*) and female offspring (B: r^2= 0.195, *P<0.05*). There was a significant negative relationship between the expression of SREBP-1c mRNA in the retroperitoneal adipose tissue and fat mass in the retroperitoneal fat depot in females (D: r^2= -0.202, *P<0.05*) and a trend towards a negative relationship in male offspring (C: r^2= -0.206, *P=0.05*).

Table 4.3 The relationship between the normalised expression of adipogenic and lipogenic genes in the subcutaneous and retroperitoneal adipose tissues and plasma glucose, NEFA, insulin and leptin concentrations, independent of treatment groups, in male and female offspring at 3 weeks of age.

Subcutaneous adipose tissue	Glucose		NEFA		Insulin		Leptin	
	Male	Female	Male	Female	Male	Female	Male	Female
SREBP-1c	ns	ns	ns	ns	r^2 = - 0.65, *P< 0.01*	ns	ns	ns
PPAR-γ	ns	ns	ns	ns	ns	ns	ns	r^2 = 0.39, *P< 0.01*
G3PDH	ns	ns	ns	ns	ns	ns	ns	r^2 = 0.26, *P< 0.05*
FAS	ns	r^2 = 0.19, *P=0.056*	ns	ns	ns	ns	ns	ns
Adiponectin	ns	ns	ns	ns	ns	ns	ns	ns
Leptin	ns	r^2 = 0.23, *P< 0.05*	ns	ns	r^2 = 0.58, *P< 0.01*	ns	r^2 = 0.40, *P< 0.01*	r^2 = 0.55, *P< 0.001*
Retroperitoneal adipose tissue								
SREBP-1c	ns	ns	ns	ns	ns	ns	ns	ns
PPAR-γ	ns	ns	ns	ns	ns	ns	ns	ns
G3PDH	ns	ns	ns	ns	ns	ns	r^2 = 0.22, *P< 0.05*	r^2 = 0.32, *P< 0.01*
FAS	ns	ns	ns	ns	ns	ns	ns	ns
Adiponectin	r^2 = 0.21, *P< 0.05*	ns	ns	ns	ns	ns	ns	r^2 = 0.38, *P< 0.01*
Leptin	ns	ns	ns	ns	r^2 = 0.51, *P< 0.05*	ns	ns	r^2 = 0.56, *P< 0.001*

4.3.1.2 *PPAR-γ mRNA expression*

There was no effect of either prenatal or postnatal exposure to the cafeteria diet on PPAR-γ mRNA expression in retroperitoneal adipose tissue in either male or female offspring at 3 weeks of age (Figure 4.3 C, D). The relative expression of PPAR-γ in the subcutaneous adipose tissue was significantly higher in female, but not in male, offspring suckled by CAF dams, independent of whether they were born to a Control or CAF dam (Figure 4.3 A, B).

There was no relationship between the expression of PPAR-γ in either the retroperitoneal or the subcutaneous depot and the mass of the respective fat depot at 3 weeks of age in males. In females, however, the expression of PPAR-γ in the subcutaneous adipose tissue tended (r^2= 0.182, *P=0.054*) to be positively related to the relative mass of this depot.

The expression of PPAR-γ mRNA in the subcutaneous and retroperitoneal adipose tissue was not related to plasma concentrations of glucose, NEFA or insulin in either male or female offspring at 3 weeks of age (Table 4.3). There was, however, a positive relationship between the PPAR-γ mRNA expression in the subcutaneous adipose tissue and plasma leptin concentrations in female offspring, but not in males, at this time (Table 4.3). There was no relationship between the expression of PPAR-γ in the retroperitoneal adipose tissue and plasma concentrations of leptin in either males or in females (Table 4.3).

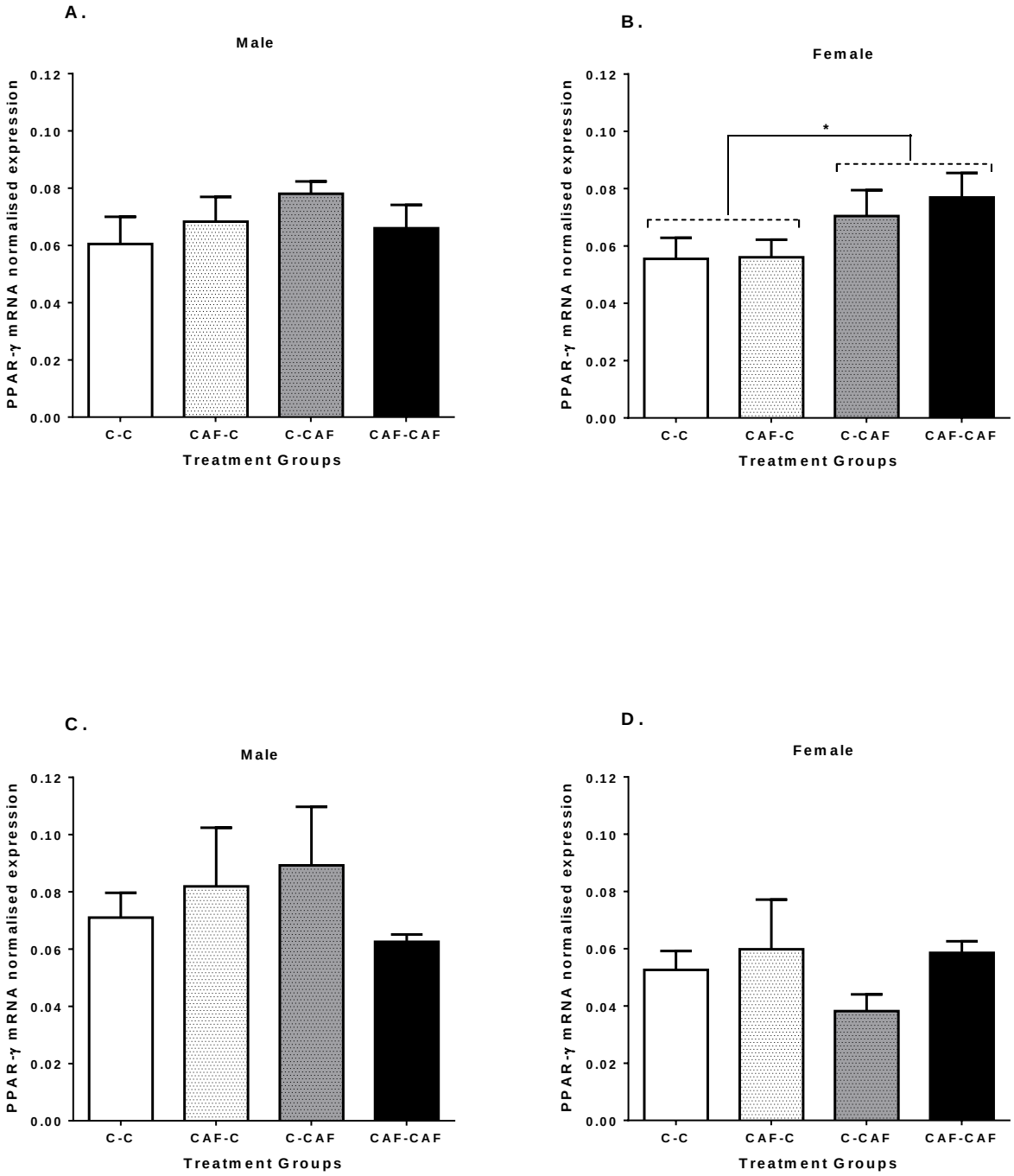


Figure 4.3 The relative expression of PPAR-γ mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

4.3.1.3 *G3PDH and FAS mRNA expression*

There was no effect of either prenatal or postnatal exposure to the cafeteria diet on G3PDH or FAS mRNA expression in the subcutaneous or retroperitoneal depots at 3 weeks of age in either male or female offspring (Figure 4.4 A, B, C& D; Figure 4.5 A, B, C & D).

Neither G3PDH nor FAS mRNA expression in the retroperitoneal or subcutaneous fat was related to the relative mass of these respective fat depots at 3 weeks of age in either male or female offspring. There was, however, a positive relationship between G3PDH mRNA expression in the retroperitoneal adipose tissue and circulating leptin concentrations in the male offspring (Table 4.3) and G3PDH mRNA expression in both the subcutaneous and retroperitoneal adipose tissue and circulating leptin concentrations in the female offspring at 3 weeks of age (Table 4.3). There was no relationship between the G3PDH expression in subcutaneous adipose tissue and circulating leptin concentrations in the male offspring at 3 weeks of age (Table 4.3). There was also no relationship between the expression of G3PDH in subcutaneous or retroperitoneal adipose tissue and circulating glucose, NEFA or insulin concentrations in either males or females.

There was no relationship between FAS mRNA expression in either adipose depot and circulating NEFA, insulin or leptin concentrations in either male or in female offspring at 3 weeks of age (Table 4.3). However, in female offspring, but not in males, FAS mRNA expression in subcutaneous adipose tissue tended (r^2= 0.19, *P=0.056*) to be positively correlated with circulating glucose concentrations (Table 4.3). There was no relationship between FAS mRNA expression in retroperitoneal adipose tissue and circulating glucose concentrations in either male or female offspring at 3 weeks of age (Table 4.3).

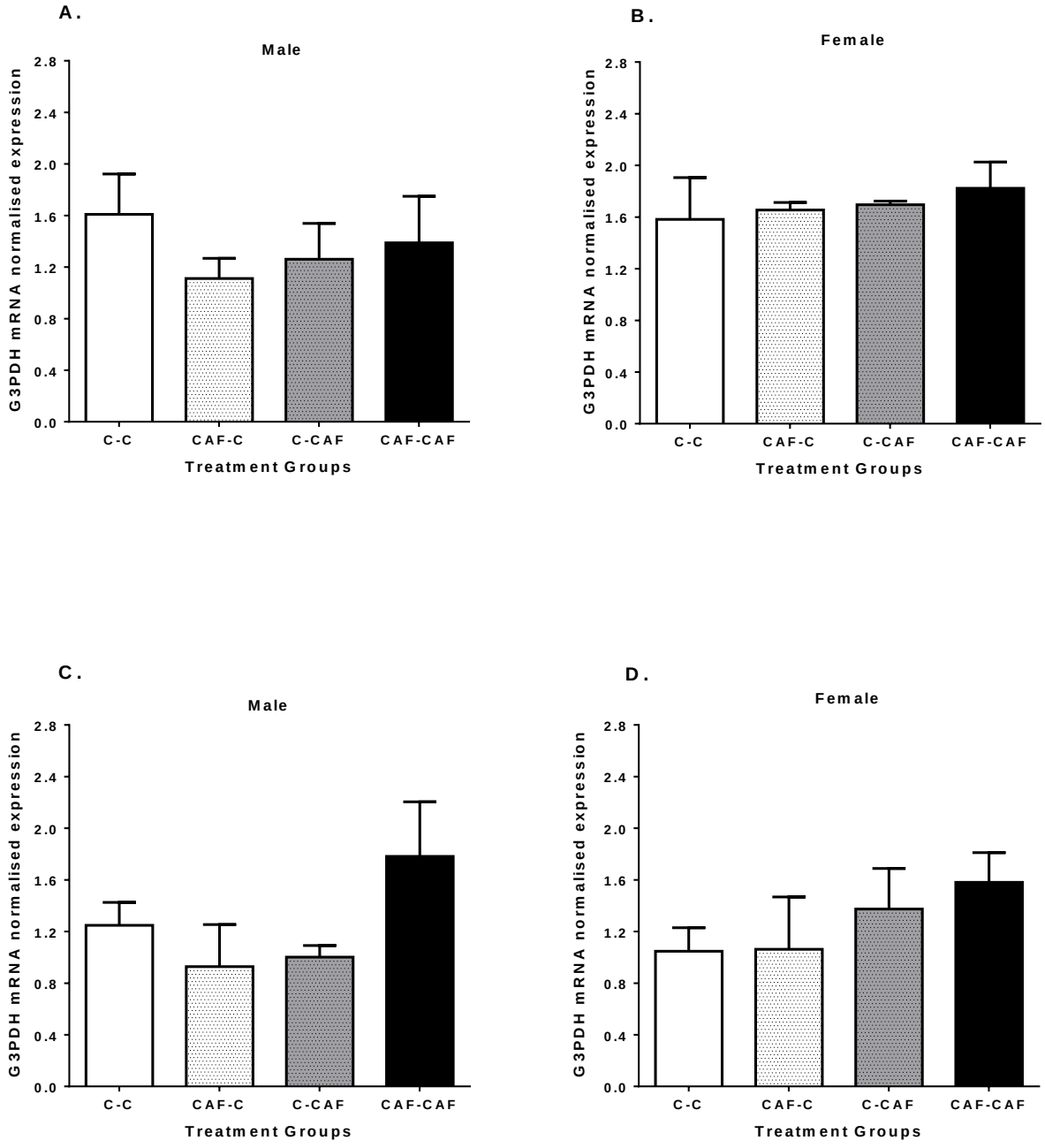


Figure 4.4 The relative expression of G3PDH mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 weeks of age. Values are expressed as mean ± SEM.

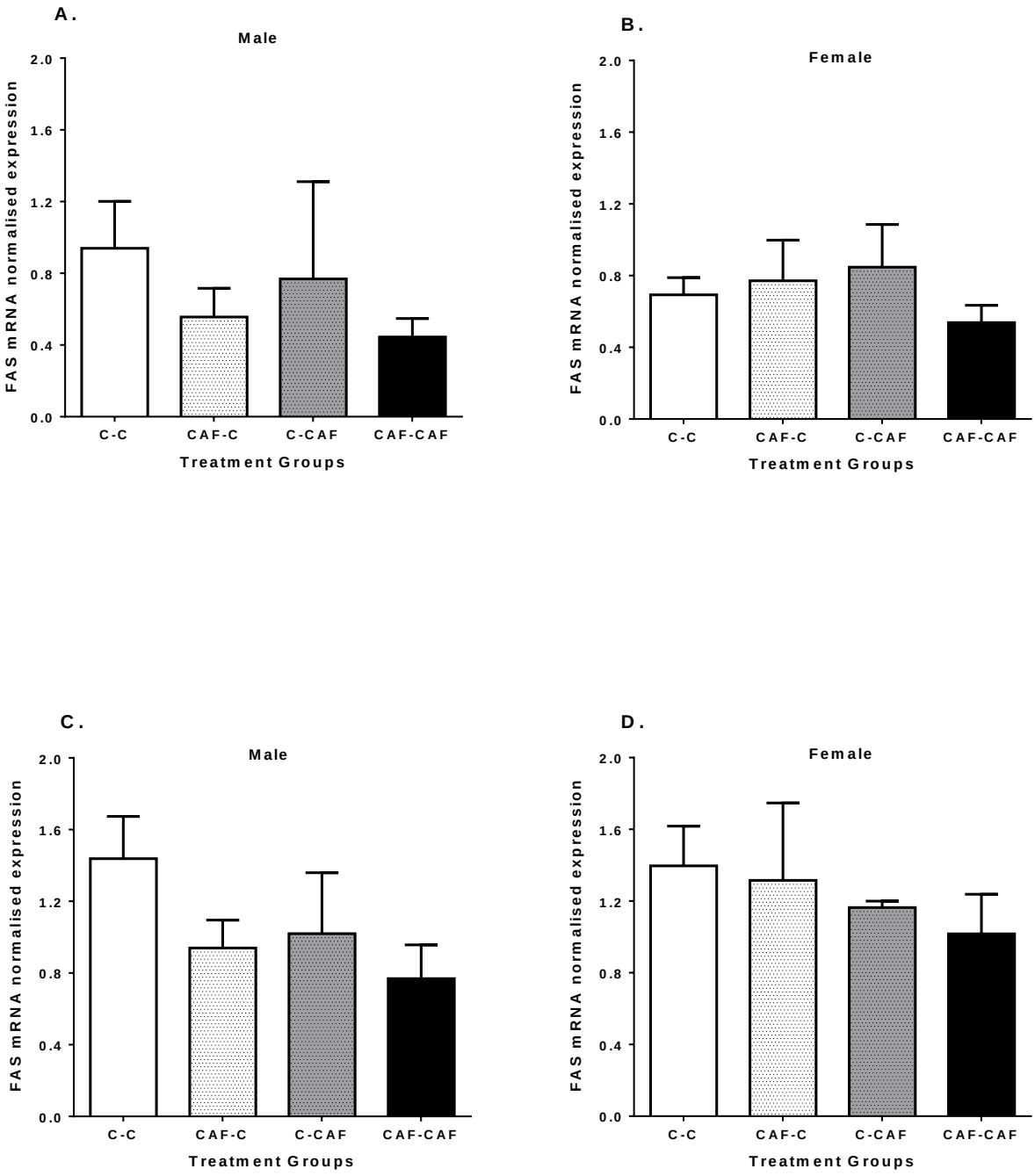


Figure 4.5 The relative expression of FAS mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 weeks of age. Values are expressed as mean ± SEM.

4.3.1.4 *Adiponectin mRNA expression*

At 3 weeks of age, the relative expression of adiponectin in the retroperitoneal adipose tissue was higher in both male and female offspring suckled by CAF dams compared to controls (Figure 4.6 C, D). In the subcutaneous adipose tissue, adiponectin mRNA expression was also higher in female, but not in male offspring, suckled by a CAF dam compared to those suckled by a control dam (Figure 4.6 A, B).

When data from all the groups were combined there was a positive correlation between the expression of adiponectin mRNA in the retroperitoneal adipose tissue and relative mass of this depot in both male and female offspring (Figure 4.7 C, D). In females, but not in males, adiponectin mRNA expression in the subcutaneous adipose tissue was also positively correlated with the relative mass of this depot (Figure 4.7 A, B).

The expression of adiponectin mRNA in the subcutaneous adipose tissue at 3 weeks of age was not related to plasma concentrations of glucose, NEFA, insulin or leptin in either males or females (Table 4.3). There was, however, a significant positive correlation between the expression of adiponectin mRNA in the retroperitoneal adipose tissue and plasma glucose concentrations in the male offspring (Table 4.3). There was also a significant positive relationship between the expression of adiponectin in the retroperitoneal adipose tissue and circulating leptin concentrations in females, but not in males, at 3 weeks of age (Table 4.3). There was no relationship between the expression of adiponectin mRNA in the retroperitoneal adipose tissue and circulating NEFA or insulin concentrations in either males or females at 3 weeks of age.

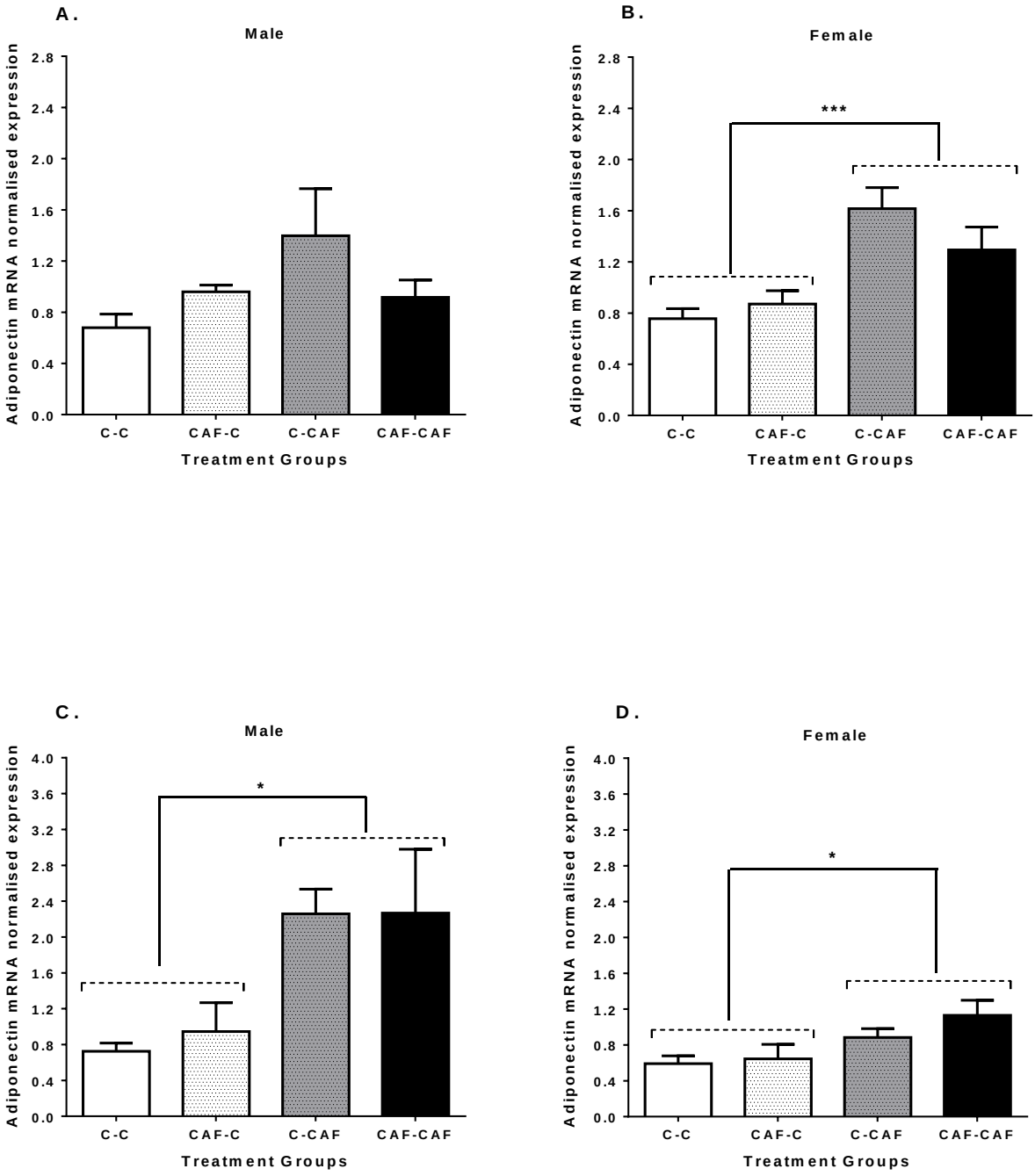


Figure 4.6 The relative expression of adiponectin mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05* and *** denotes significance at *P<0.001*.

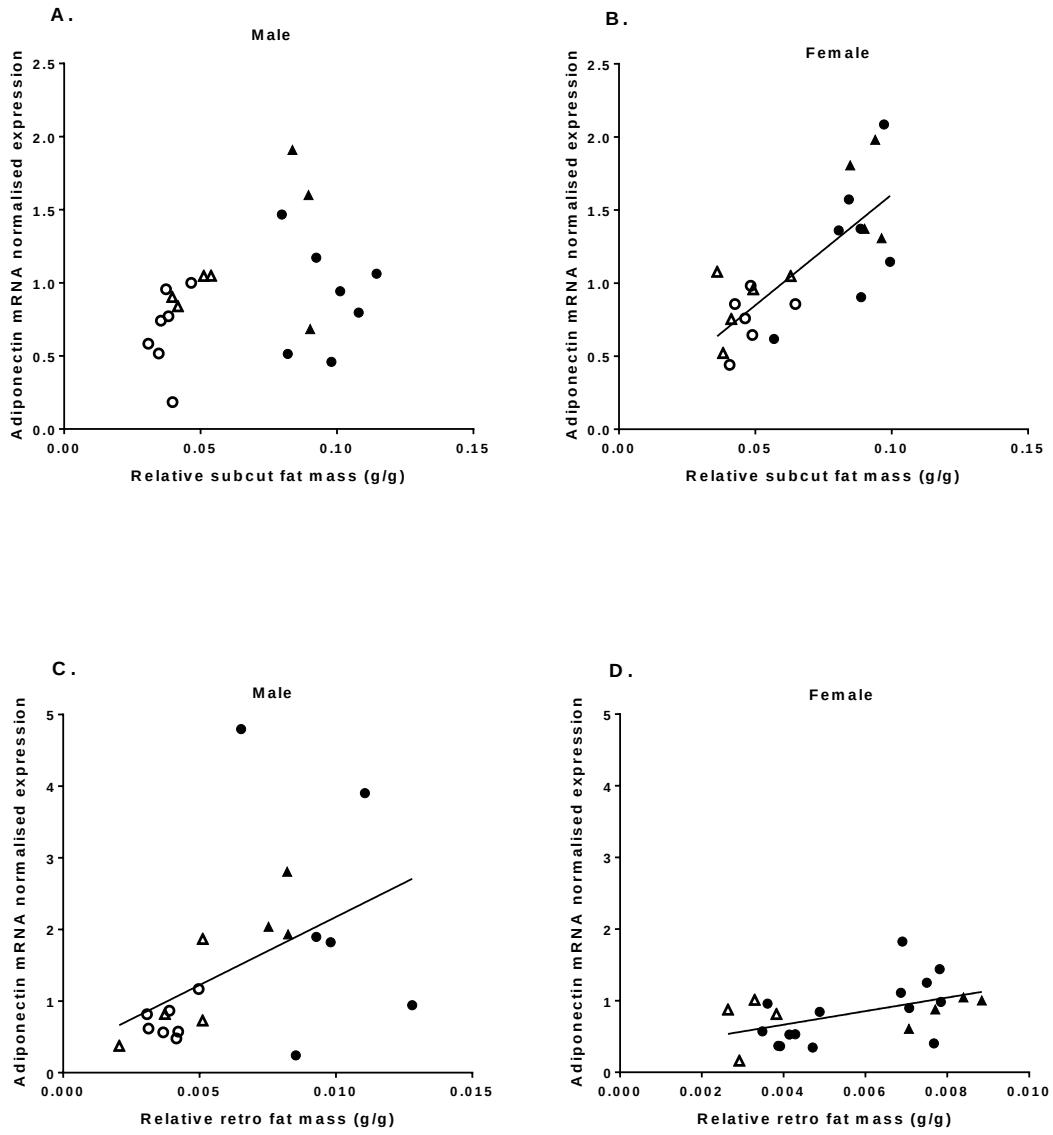


Figure 4.7 The relationship between adiponectin mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male (A) and female (B) offspring and the relationship between adiponectin mRNA expression in retroperitoneal adipose tissue and the relative mass of this fat depot in male (C) and female (D) offspring at 3 weeks of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of adiponectin mRNA in the retroperitoneal adipose tissue and relative mass of this fat depot in both male (C: r^2= 0.222, *P<0.05*) and female offspring (D: r^2= 0.245, *P<0.05*). There was also a significant positive relationship between the expression of adiponectin mRNA in the subcutaneous adipose tissue and relative subcutaneous fat mass in female offspring at 3 weeks of age. (B: r^2= 0.585, *P<0.001*).

4.3.1.5 *Leptin mRNA expression*

Pups suckled by CAF dams had significantly increased leptin mRNA expression in both subcutaneous and retroperitoneal adipose depots in both male and female offspring at 3 weeks of age (Figure 4.8 A, B, C& D). Leptin mRNA expression in both subcutaneous and retroperitoneal adipose tissue was also positively correlated with the relative mass of the respective fat depot in both male and female offspring when data from all treatment groups were combined (Figure 4.9 A, B, C& D).

The expression of leptin mRNA in the subcutaneous adipose tissue was positively related to circulating leptin concentrations in both male and female offspring (Table 4.3), while leptin mRNA expression in the retroperitoneal adipose tissue was positively related to circulating leptin concentrations in female offspring only (Table 4.3). In males, however, there was a positive correlation between the expression of leptin in both the subcutaneous and retroperitoneal adipose tissues and plasma insulin concentrations at 3 weeks of age, however this relationship was not present in females (Table 4.3). Conversely, the expression of leptin mRNA in subcutaneous adipose tissue was positively correlated with circulating glucose concentrations in females, but not in males, at 3 weeks of age (Table 4.3). There was no relationship between the expression of leptin mRNA in the retroperitoneal adipose tissue and plasma glucose concentrations in either males or females (Table 4.3). There was also no relationship between the expression of leptin mRNA in either depot and plasma concentrations of NEFA in either males or in females (Table 4.3).

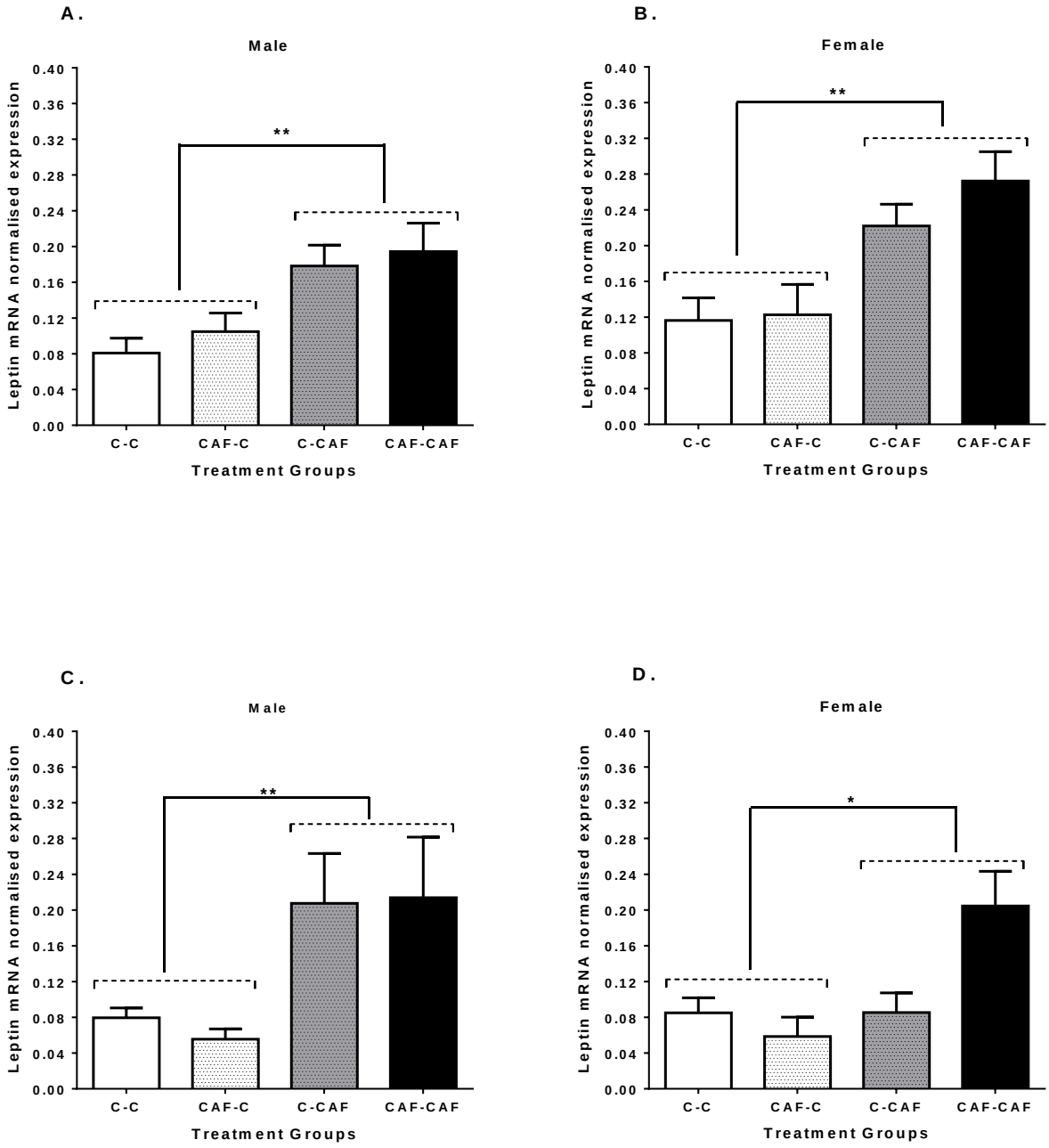


Figure 4.8 The relative expression of leptin mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05* and ** denotes significance at *P<0.01*.

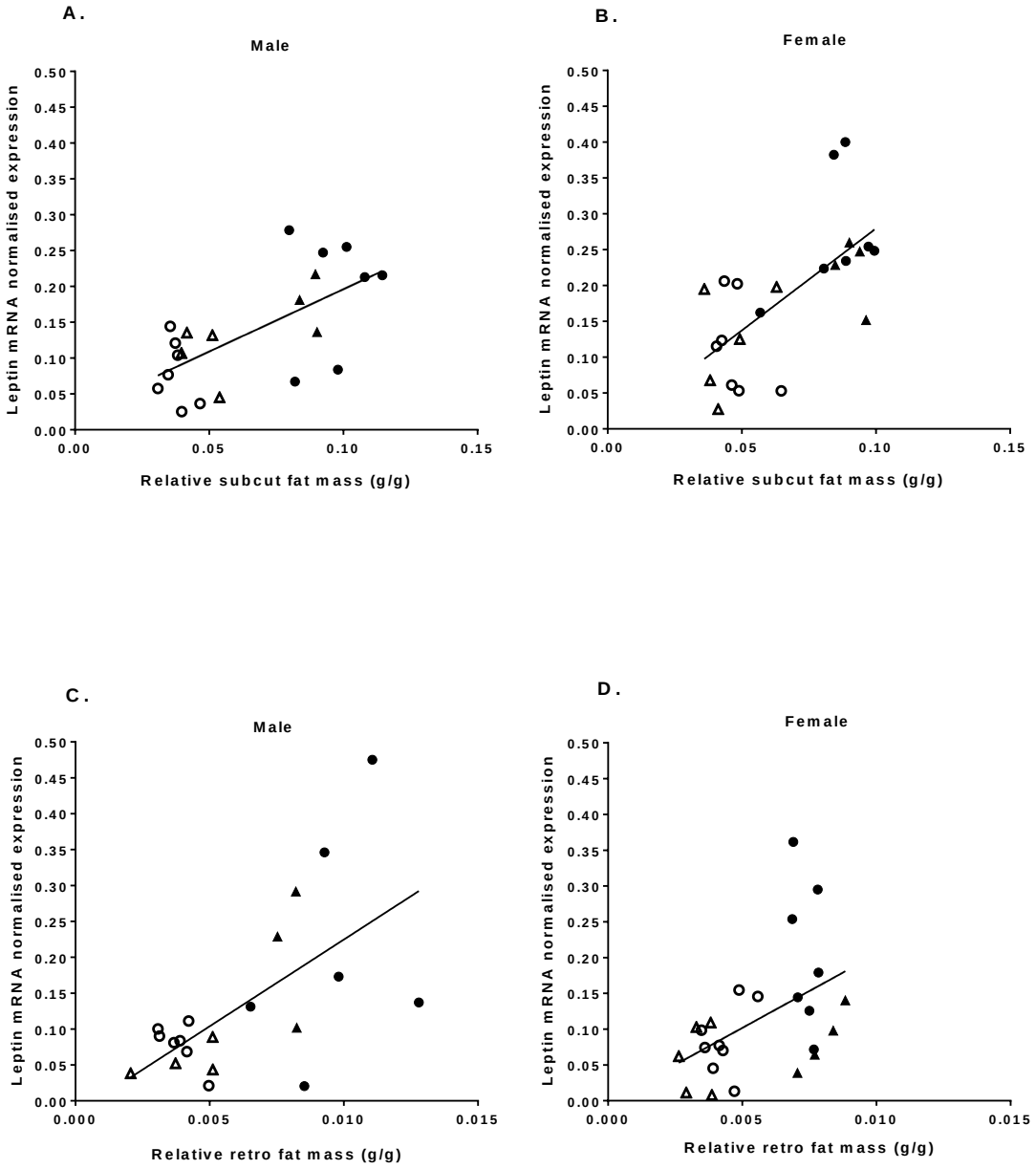


Figure 4.9 The relationship between leptin mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male (A) and female (B) offspring and the relationship between leptin mRNA expression in retroperitoneal adipose tissue and relative mass of this fat depot in male (C) and female (D) offspring at 3 weeks of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of leptin mRNA in both the subcutaneous and retroperitoneal adipose tissue and the relative mass of the respective fat depot in both male and female offspring. (A: r^2= 0.424, *P<0.01*, B: r^2= 0.443, *P<0.01*, C: r^2= 0.381, *P<0.01*, D: r^2= 0.221, *P<0.05*).

4.3.2 Expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal adipose tissue at 6 weeks of age

4.3.2.1 *SREBP-1c mRNA expression*

At 6 weeks of age, when all offspring had been fed on a control diet for 3 weeks post weaning, the relative expression of SREBP-1c in subcutaneous adipose tissue was not different between the groups in males, but was lower in the female offspring suckled by a CAF dam compared to those suckled by a Control dam, independent of prenatal diet exposure (Figure 4.10 A, B). There were no differences between the groups in the expression of SREBP-1c mRNA in retroperitoneal adipose tissue in either male or female offspring at this time point (Figure 4.10 C, D).

There was no relationship between the expression of SREBP-1c mRNA in either subcutaneous or retroperitoneal adipose depots and the relative mass of the respective depot in either male or female offspring. The expression of SREBP-1c mRNA in retroperitoneal tissue was, however, negatively related to circulating leptin concentrations in females, but not in males (Table 4.4). There was no significant correlation between the expression of SREBP-1c in either subcutaneous or retroperitoneal adipose tissue and plasma glucose, NEFA or insulin concentrations in either male or in the female offspring at 6 weeks of age (Table 4.4).

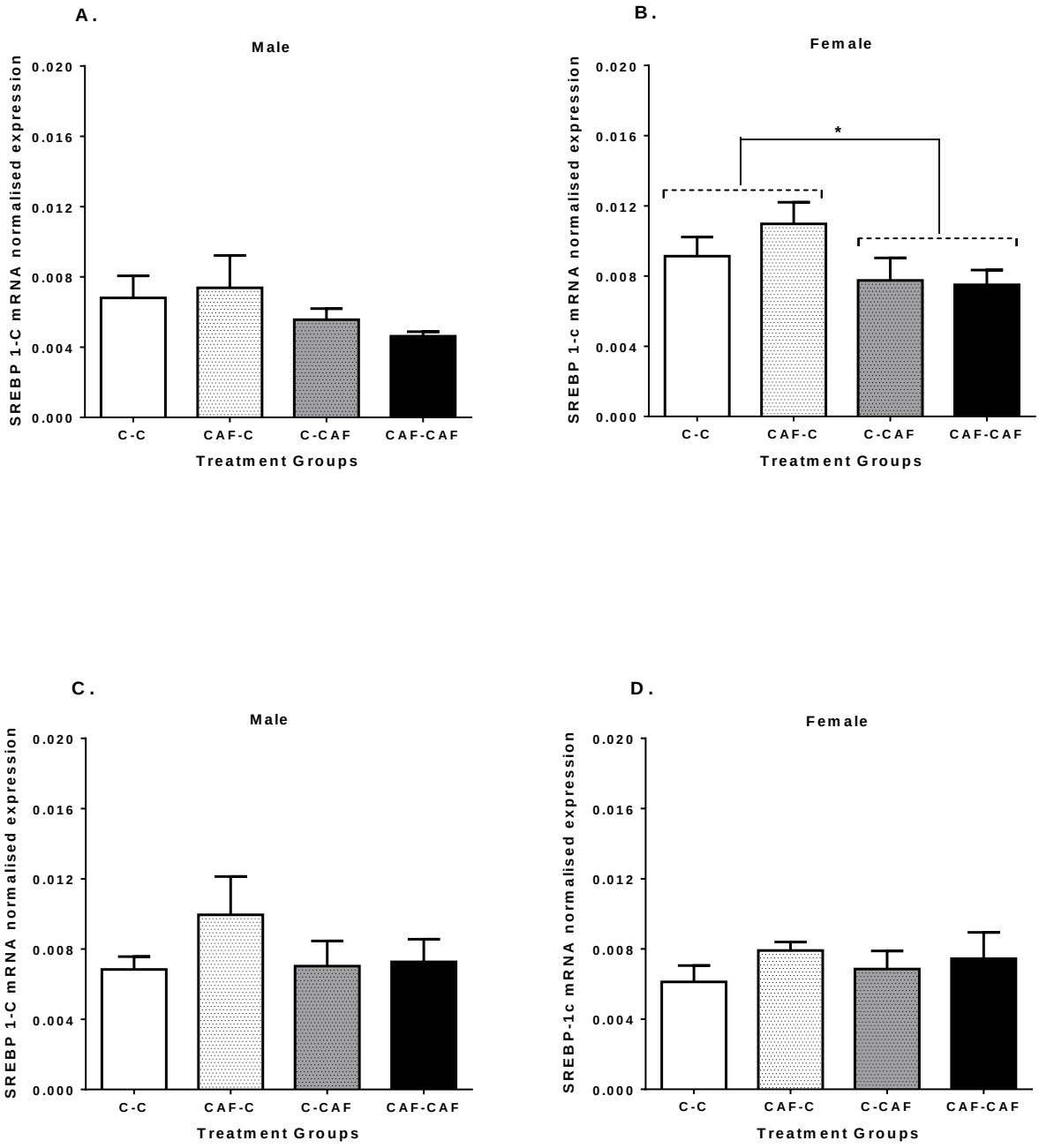


Figure 4.10 The relative expression of SREBP-1c mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 6 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

Table 4.4 The relationship between the normalised expression of adipogenic and lipogenic genes in the subcutaneous and retroperitoneal adipose tissues and plasma glucose, NEFA, insulin and leptin concentrations, independent of treatment group, in male and female offspring at 6 weeks of age.

Subcutaneous adipose tissue	Glucose		NEFA		Insulin		Leptin	
	Male	Female	Male	Female	Male	Female	Male	Female
SREBP-1c	ns	ns	ns	ns	ns	ns	ns	ns
PPAR-γ	ns	ns	ns	r^2 = - 0.22, *P< 0.05*	ns	ns	ns	ns
G3PDH	ns	ns	ns	ns	ns	ns	r^2 = 0.24, *P< 0.05*	ns
FAS	ns	ns	ns	ns	ns	ns	r^2 = 0.45, *P< 0.01*	ns
Adiponectin	ns	ns	ns	ns	ns	ns	ns	ns
Leptin	ns	ns	ns	ns	r^2 = 0.41, *P< 0.01*	ns	r^2 = 0.30, *P< 0.05*	ns
Retroperitonal adipose tissue								
SREBP-1c	ns	ns	ns	ns	ns	ns	ns	r^2 = - 0.29, *P< 0.01*
PPAR-γ	ns	ns	ns	ns	ns	ns	ns	ns
G3PDH	ns	ns	ns	ns	ns	ns	ns	ns
FAS	ns	ns	ns	ns	ns	ns	ns	ns
Adiponectin	ns	ns	ns	ns	ns	ns	ns	r^2 = 0.20, *P< 0.05*
Leptin	ns	ns	ns	ns	ns	ns	r^2 = 0.19, *P= 0.06*	r^2 = 0.26, *P< 0.05*

4.3.2.2 *PPAR-γ mRNA expression*

There was no difference in PPAR-γ mRNA expression in the subcutaneous adipose tissue between groups either in male or in female offspring at 6 weeks of age (Figure 4.11 A, B). There was also no difference in the relative expression of PPAR-γ mRNA in retroperitoneal adipose tissue between the groups in the male offspring (Figure 4.11 C). In females, however, there was an interaction between the effects of exposure to the cafeteria diet during the prenatal and the suckling periods, such that the expression of PPAR-γ in the retroperitoneal fat depot was decreased in pups born to a CAF dam suckled by a Control dam compared to those pups both born to and suckled by a Control dam (Figure 4.11 D).

There was no relationship between the expression of PPAR-γ mRNA in either the subcutaneous or retroperitoneal adipose tissue and relative mass of the respective fat depot in either male or female offspring.

The expression of PPAR-γ mRNA in the subcutaneous and retroperitoneal adipose tissue was not related to plasma concentrations of glucose, insulin or leptin in either male or female offspring at 6 weeks of age (Table 4.4). There was however, a negative correlation between the expression of PPAR-γ mRNA in the subcutaneous adipose tissue and plasma NEFA concentrations in female offspring (Table 4.4). There was no relationship between the expression of PPAR-γ mRNA in the retroperitoneal adipose tissue and plasma concentrations of NEFA in either male or female offspring at 6 weeks of age (Table 4.4).

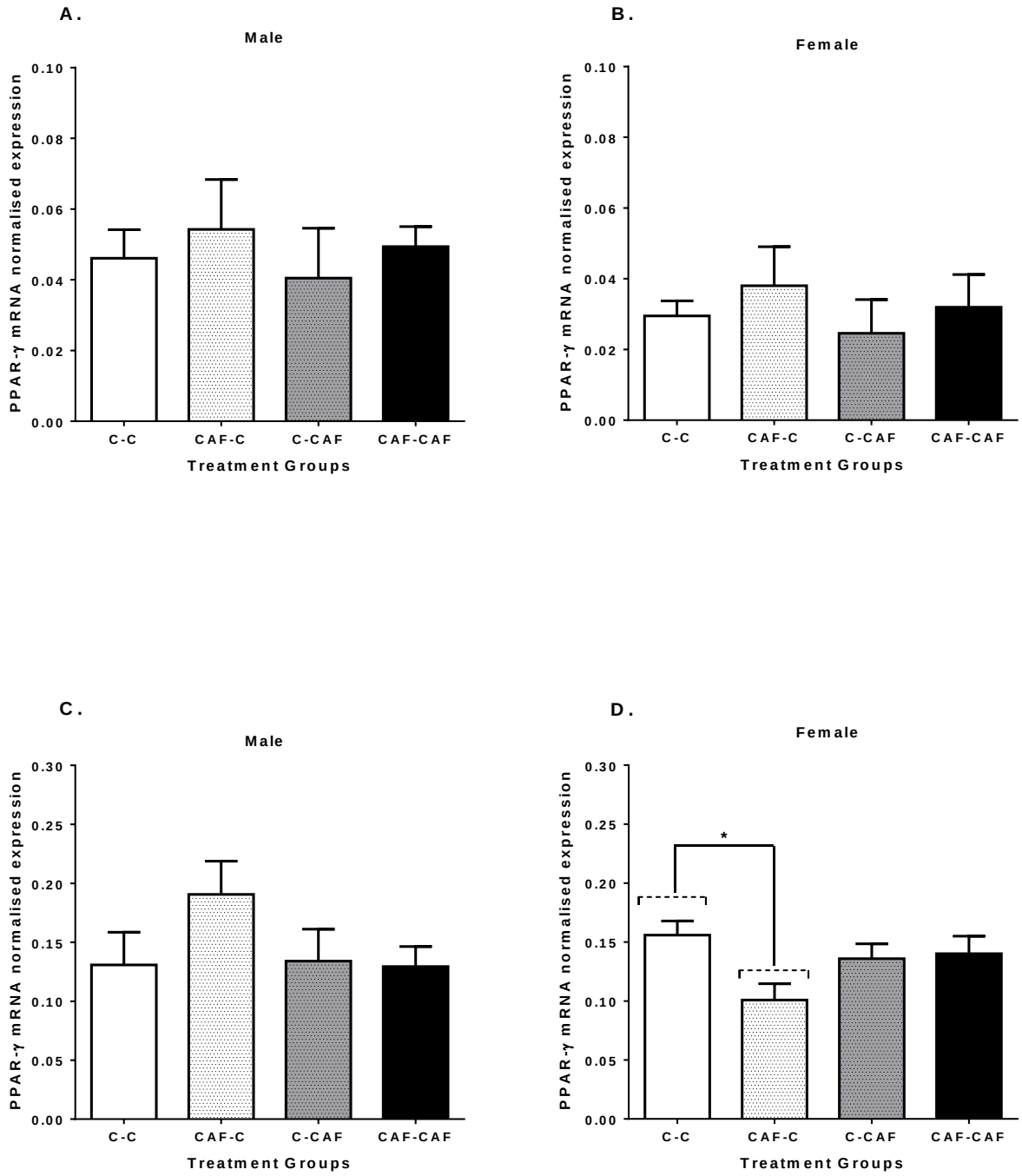


Figure 4.11 The relative expression of PPAR-γ mRNA in subcutaneous (A, B) and retroperitoneal (C, D) adipose tissue in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 6 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

4.3.2.3 *G3PDH and FAS mRNA expression*

There was no effect of either prenatal or postnatal exposure to a cafeteria diet on G3PDH and FAS mRNA expression in the retroperitoneal depot in females or the subcutaneous depot in either male or female offspring at 6 weeks of age (Figure 4.12 A, B, D; Figure 4.13 A, B, D). In males, however, there was a significant interaction between the effects of prenatal and postnatal exposure to the cafeteria diet in relation to both G3PDH and FAS mRNA expression, such that pups born to a CAF dam who were suckled by a Control dam exhibited significantly higher G3PDH and FAS mRNA expression in the retroperitoneal depot compared to those pups both born to and suckled by a Control dam (Figure 4.12 C; Figure 13 C).

Neither G3PDH or FAS mRNA expression were related to the relative weight of the fat depot in either retroperitoneal or subcutaneous adipose tissue at 6 weeks of age in either male or female offspring. There was, however, a positive relationship between both G3PDH and FAS mRNA expression in the subcutaneous adipose tissue and circulating leptin concentrations in the male offspring, but not in females, at 6 weeks of age (Table 4.4). There was no relationship between G3PDH or FAS mRNA expression in the retroperitoneal adipose tissue and circulating leptin concentrations in either male or female offspring (Table 4.4). There was also no relationship between the expression of G3PDH and FAS mRNA expression in either depot and circulating glucose, NEFA or insulin concentrations in either males or females (Table 4.4).

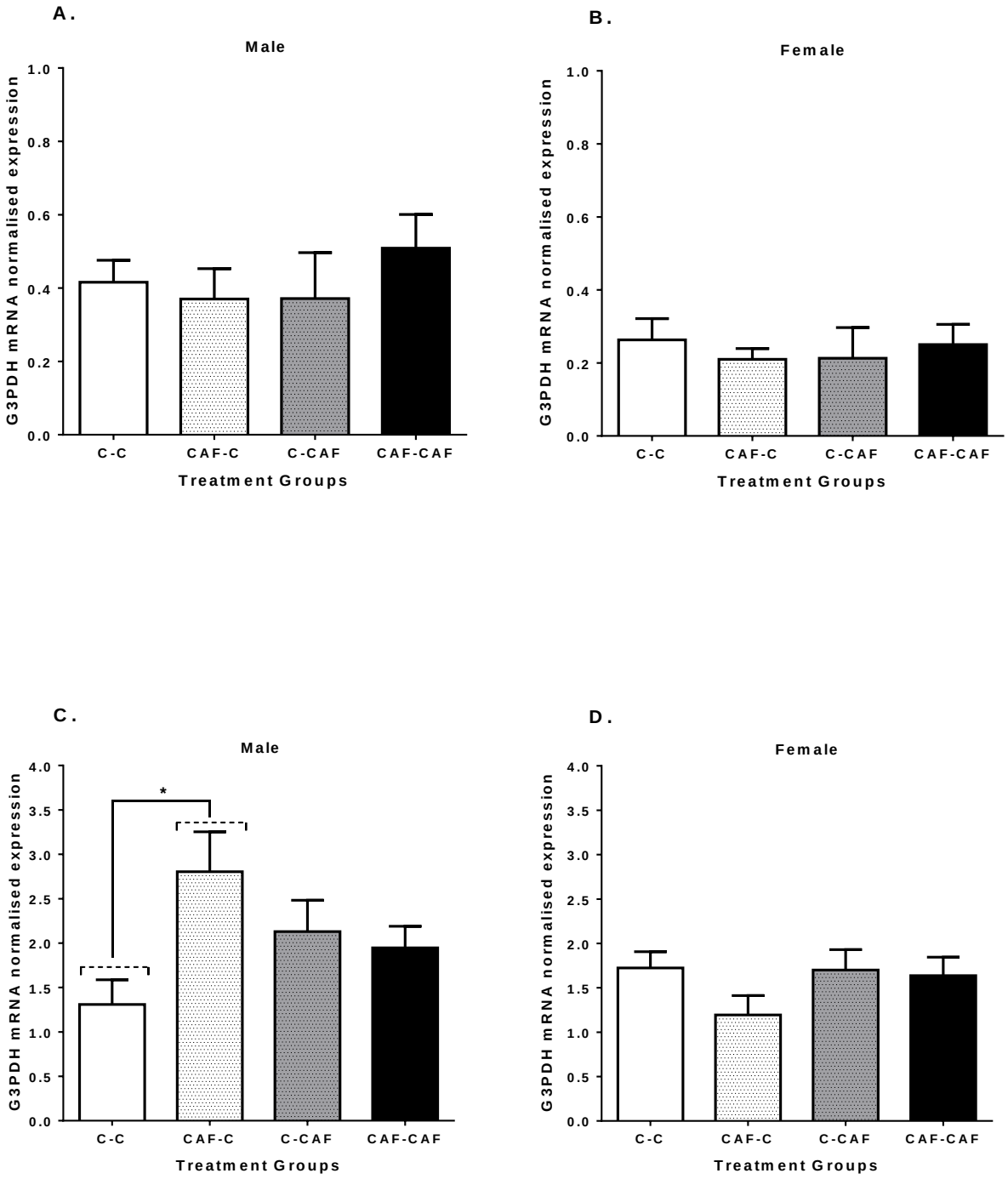


Figure 4.12 The relative expression of G3PDH mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 6 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

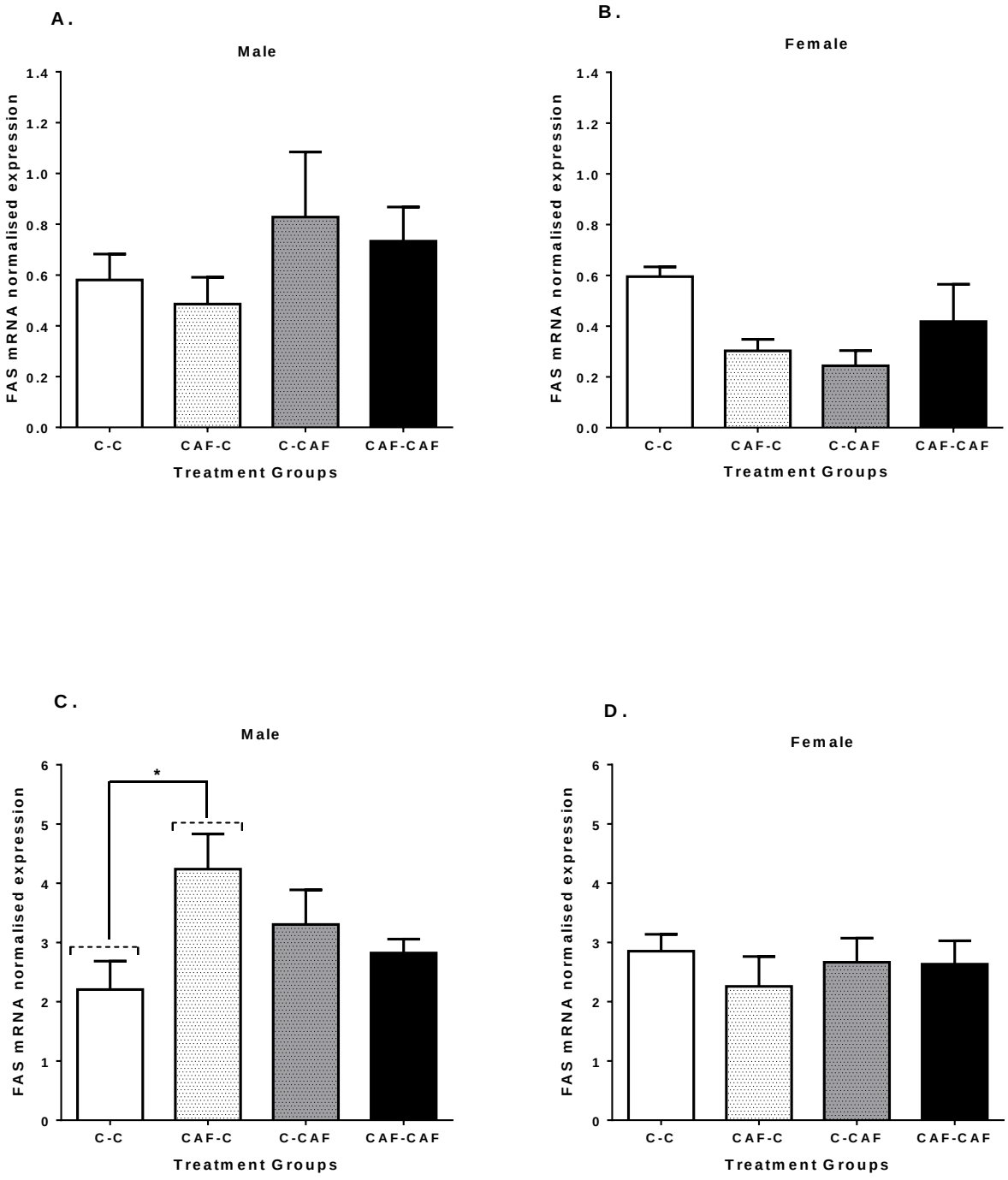


Figure 4.13 The relative expression of FAS mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 6 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

4.3.2.4 *Adiponectin mRNA expression*

At 6 weeks of age, there was no difference in the relative expression of adiponectin mRNA in the subcutaneous adipose tissue between groups in either males or females (Figure 4.14 A, B). In the retroperitoneal adipose tissue, however, adiponectin mRNA expression was significantly lower in female offspring who were born to a CAF dam compared to those born to a control dam (Figure 4.14 D), independent of dietary exposure during the suckling period. There were no differences in adiponectin mRNA expression in the retroperitoneal depot between the groups in male offspring (Figure 4.14 C).

There was no relationship between the expression of adiponectin mRNA in the subcutaneous adipose tissue and the relative mass of this depot in either males or females at 6 weeks of age (Figure 4.15 A, B). In female offspring, however, adiponectin mRNA expression in the retroperitoneal adipose tissue was positively related to the relative mass of this depot, as it was at 3 weeks (Figure 4.15 D). This relationship was not present in male offspring (Figure 4.15 C).

The expression of adiponectin mRNA in the subcutaneous adipose tissue was not related to plasma concentrations of glucose, NEFA, insulin or leptin in either males or females at 6 weeks of age (Table 4.4). There was also no relationship between the expression of adiponectin in the retroperitoneal adipose tissue and circulating glucose, NEFA or insulin concentrations in either males or females. There was, however, a significant positive correlation between the expression of adiponectin mRNA in the retroperitoneal adipose tissue and circulating leptin concentrations in the female offspring, but not in males (Table 4.4).

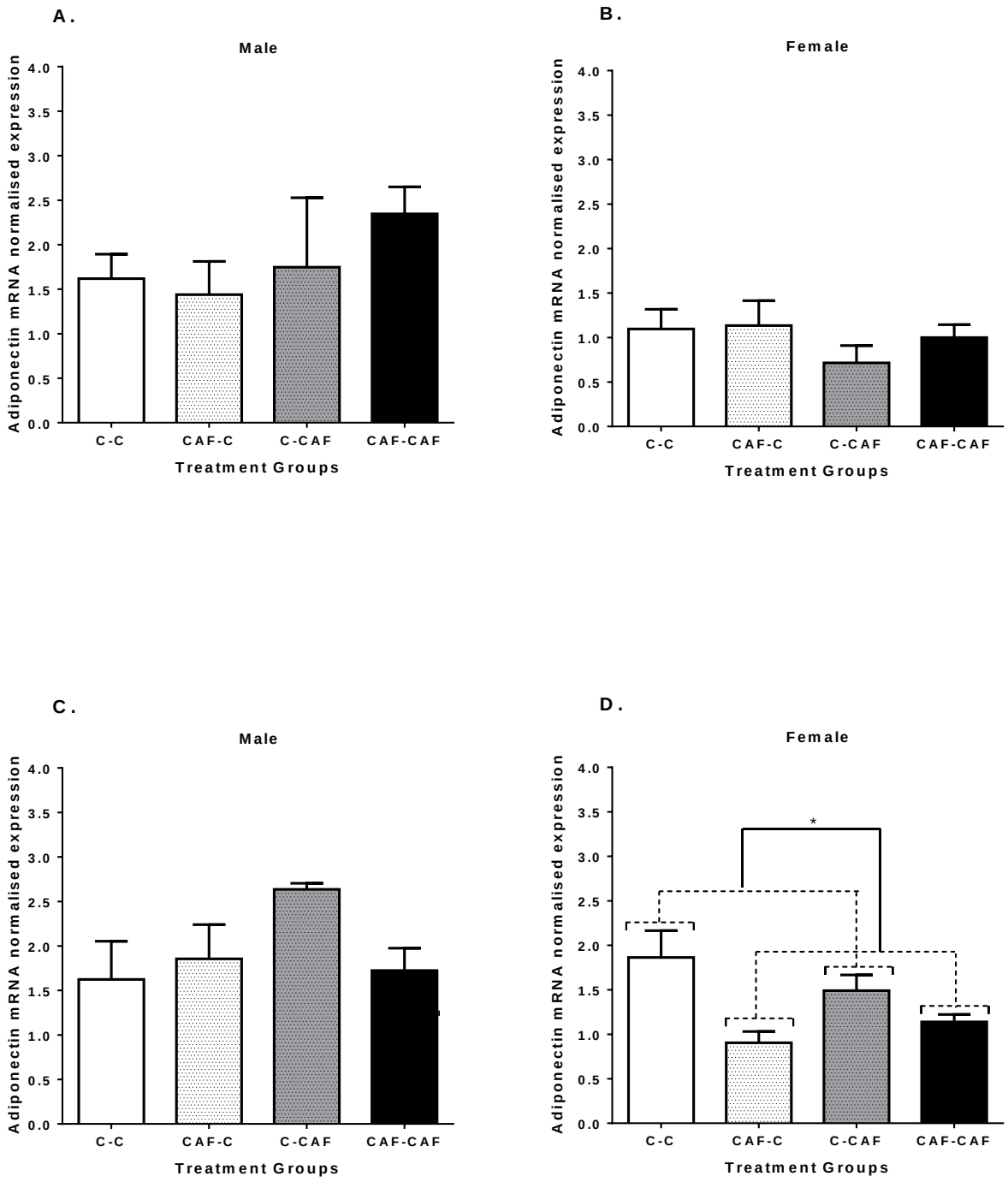


Figure 4.14 The relative expression of adiponectin mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 6 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

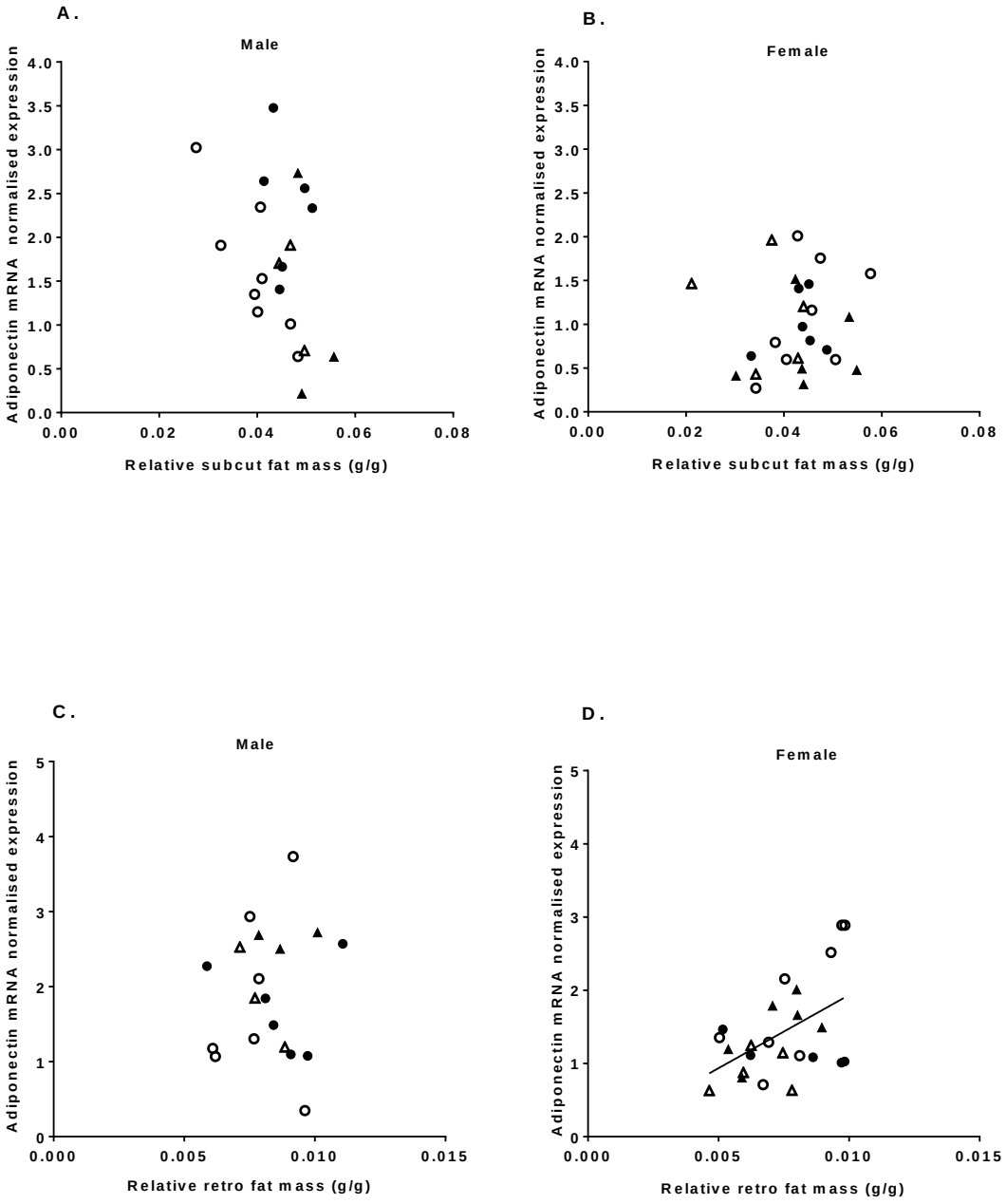


Figure 4.15 The relationship between adiponectin mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male (A) and female (B) offspring and the relationship between adiponectin mRNA expression in retroperitoneal adipose tissue and relative mass of this fat depot in male (C) and female (D) offspring at 6 weeks of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of adiponectin mRNA in the retroperitoneal adipose tissue and the relative mass of this fat depot in female offspring at 6 weeks of age. (D: r^2= 0.251, *P<0.05*).

4.3.2.5 *Leptin mRNA expression*

There was no difference in the relative expression of leptin mRNA in the subcutaneous adipose tissue between groups in either male or female offspring at 6 weeks of age (Figure 4.16 A, B). There was also no difference in the relative expression of leptin mRNA in the retroperitoneal adipose tissue between the groups in male offspring at 6 weeks of age (Figure 4.16 C). However, leptin mRNA expression in the retroperitoneal adipose tissue was significantly lower in female offspring who were born to a CAF dam compared to those born to a control dam, independent of the diet they were exposed to during the suckling period (Figure 4.16 D).

When data from all groups were combined there was a significant positive relationship between the expression of leptin mRNA in the retroperitoneal adipose tissue and the relative mass of this fat depot in females, but not in males (Figure 4.17 C, D). There was no significant relationship between the expression of leptin mRNA in the subcutaneous adipose tissue and relative mass of this depot in either males or females (Figure 4.17 A, B).

The expression of leptin mRNA in the subcutaneous adipose tissue was positively correlated with circulating leptin concentrations in male offspring (Table 4.4). In females, however, circulating leptin concentrations were positively correlated with leptin mRNA expression in the retroperitoneal adipose tissue (Table 4.4), and this relationship also tended (r^2= 0.19, *P=0.06*) towards significance in males (Table 4.4). There was no relationship between the expression of leptin mRNA in either the subcutaneous or retroperitoneal adipose tissue and plasma concentrations of glucose, NEFA or insulin in either males or in females at 6 weeks of age (Table 4.4).

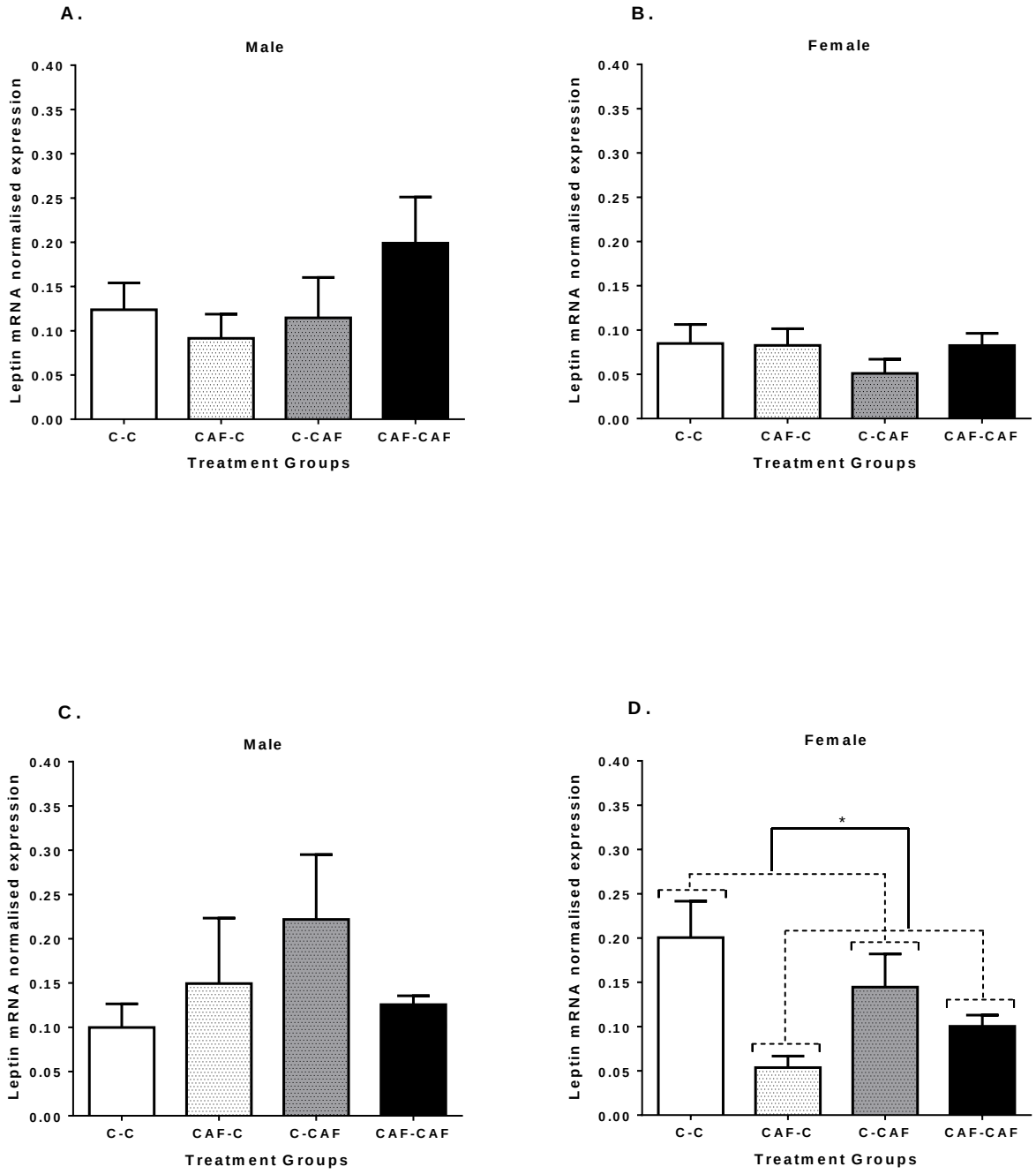


Figure 4.16 The relative expression of leptin mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 6 weeks of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

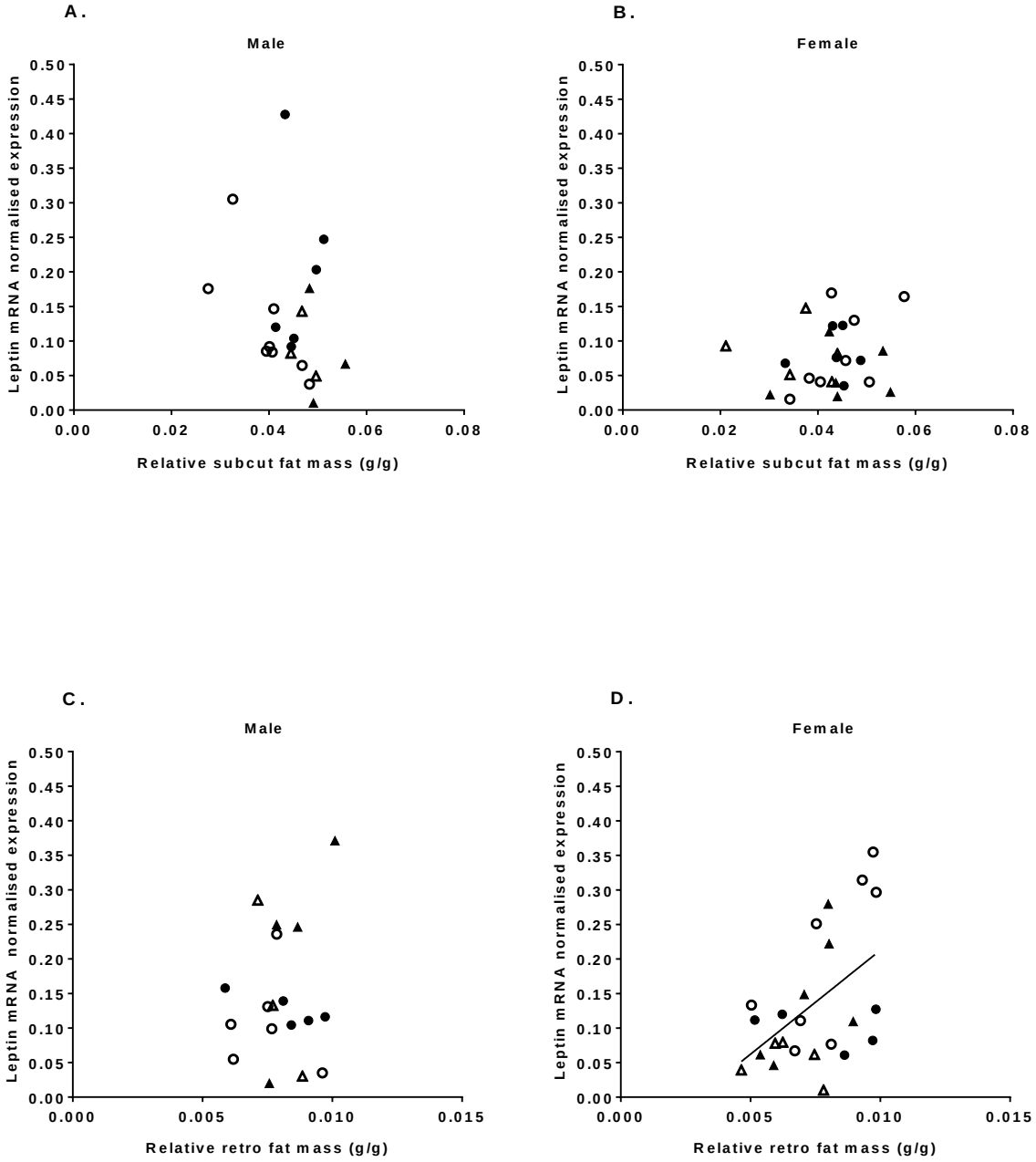


Figure 4.17 The relationship between leptin mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male (A) and female (B) offspring and the relationship between leptin mRNA expression in retroperitoneal adipose tissue and the relative mass of this fat depot in male (C) and female (D) offspring at 6 weeks of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of leptin mRNA in the retroperitoneal adipose tissue and relative retroperitoneal fat mass in female offspring at 6 weeks of age. (D: r^2= 0.257, *P<0.05*).

4.3.3 Differences in the expression of adipogenic and lipogenic genes between subcutaneous and retroperitoneal adipose tissue in the male and female offspring at 3 weeks and 6 weeks of age

4.3.3.1 *3 weeks*

In males, the expression of SREBP-1c mRNA was significantly higher in the subcutaneous compared to the retroperitoneal depot, while FAS mRNA expression was significantly higher in the retroperitoneal compared to subcutaneous fat, independent of treatment group (Table 4.5). There was no difference in the expression of PPAR-γ, G3PDH or leptin mRNA between the retroperitoneal and subcutaneous fat depots at 3 weeks of age (Table 4.5). The relative expression of adiponectin tended (*P=0.06*) to be higher in the retroperitoneal adipose tissue compared to subcutaneous adipose tissue (Table 4.5).

In females, however, mRNA expression of SREBP-1c, PPAR-γ, G3PDH, adiponectin and leptin were all significantly higher in the subcutaneous compared to the retroperitoneal fat depot, independent of treatment group (Table 4.5). Conversely, the relative expression of FAS was higher in the retroperitoneal adipose tissue compared to subcutaneous adipose tissue at 3 weeks of age (Table 4.5).

4.3.3.2 *6 weeks*

At 6 weeks of age, in male offspring, the expression of PPAR-γ, G3PDH, and FAS mRNA was higher in the retroperitoneal compared to subcutaneous fat depot, independent of treatment group (Table 4.5). There was no difference however in the expression of SREBP-1c, adiponectin or leptin mRNA between the fat depots in male offspring (Table 4.5).

In female offspring PPAR-γ, G3PDH, FAS, adiponectin and leptin mRNA expression was significantly higher in the retroperitoneal compared to subcutaneous fat, independent of treatment group (Table 4.5). Conversely, mRNA expression of SREBP-1c was higher in the subcutaneous adipose tissue compared to retroperitoneal adipose tissue, independent of treatment group (Table 4.5).

Table 4.5 The normalised expression of adipogenic and lipogenic genes between subcutaneous and retroperitoneal adipose tissue in the male and female offspring, independent of treatment groups, at 3 weeks and 6 week of age. Values are expressed as means ± SEM.* denotes significance at *P<0.05*, ** denotes significance at *P<0.01* and *** denotes significance at *P<0.001*.# denotes a trend at *P=0.06*.

3 weeks

	Male (n = 21)		Female (n = 23)	
	Subcutaneous	**Retroperitoneal**	**Subcutaneous**	**Retroperitoneal**
SREBP-1c	0.009 ± 0.0006	0.006 ± 0.0006**	0.009 ± 0.0007	0.006 ± 0.0004***
PPAR-γ	0.06 ± 0.004	0.07 ± 0.006	0.07 ± 0.004	0.05 ± 0.005 *
G3PDH	1.39 ± 0.16	1.31 ± 0.16	1.69 ± 0.12	1.26 ± 0.13*
FAS	0.68 ± 0.12	1.08 ± 0.13*	0.69 ± 0.07	1.23 ± 0.13***
Adiponectin	0.91 ± 0.09	1.46 ± 0.27#	1.11 ± 0.10	0.81 ± 0.08*
Leptin	0.14 ± 0.02	0.13 ± 0.03	0.18 ± 0.02	0.11 ± 0.03*

6weeks

	Male (n = 21)		Female (n = 25)	
	Subcutaneous	**Retroperitoneal**	**Subcutaneous**	**Retroperitoneal**
SREBP-1c	0.006 ± 0.001	0.007 ± 0.0005	0.009 ± 0.0006	0.007 ± 0.0005*
PPAR-γ	0.05 ± 0.004	0.14 ± 0.01***	0.03 ± 0.004	0.14 ± 0.007 ***
G3PDH	0.40 ± 0.04	1.86 ± 0.18***	0.25 ± 0.03	1.59 ± 0.10***
FAS	0.66 ± 0.07	2.88 ± 0.27***	0.44 ± 0.06	2.64 ± 0.18***
Adiponectin	1.83 ± 0.20	1.79 ± 0.20	0.99 ± 0.11	1.48 ± 0.14**
Leptin	0.11 ± 0.01	0.16 ± 0.03	0.08 ± 0.009	0.14 ± 0.02**

4.4 DISCUSSION

In this study, I found sex and depot specific differences in the expression of key adipogenic and lipogenic genes in the adipose tissue of rat offspring exposed to a cafeteria diet before birth and/or during the suckling period. A key finding was that the exposure to cafeteria diet during the suckling period, independent of dietary exposure before birth, resulted in an increased expression of PPAR-γ mRNA in subcutaneous fat in female offspring at weaning, suggesting that this may be responsible for driving the increased relative fat mass in these offspring at this time point. Interestingly, and contrary to our expectations, the mRNA expression of the lipogenic transcription factor SREBP-1c at weaning was down-regulated in both male and female offspring who were suckled by dams fed the cafeteria diet, independent of dietary exposure before birth, and was still down-regulated in the subcutaneous depot of female offspring at 6 weeks of age. Another important finding of this study was that adiponectin mRNA expression at weaning was significantly increased in at least one fat depot in both male and female offspring who were exposed to the maternal cafeteria diet during the suckling period. These findings suggest that exposure to a maternal cafeteria diet during the suckling period alters the expression of key adipogenic and lipogenic factors in both subcutaneous and visceral adipose depots at weaning in a sex-specific manner, and that some, but not all, of these changes persist after the pups have been fed on a nutritionally balanced chow diet for 3 weeks after weaning.

4.4.1 Impact of increased maternal nutrition during the suckling period on the expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal adipose tissue at 3 weeks and 6 weeks of age

4.4.1.1 *SREBP-1c mRNA expression*

An unexpected finding of this study was that mRNA expression of the lipogenic transcription factor, SREBP-1c, at weaning was lower in both the subcutaneous fat of males and retroperitoneal fat of female offspring who were suckled by

cafeteria-fed dams, despite the significantly higher relative fat mass in these offspring. While these findings were contrary to our expectations, given that SREBP-1c has been implicated in promoting fat storage, they are consistent with previous studies in humans [471-474] which have demonstrated that SREBP-1c mRNA level was decreased in obese subjects and negatively correlated with BMI, indicating an association between increased body weight/fat storage and the down-regulation of this transcription factor [474]. The observations from the present study are also consistent with DNA microarray studies in animal models, which demonstrated that the expression of SREBP-1c mRNA is two-to-threefold lower in white adipose tissue in obese compared to lean animals [312, 475].

One possible mechanism, as speculated by the authors of previous rodent studies [312, 475], is that the reduction in the expression of SREBP-1c in obese individuals/animals reflects down-regulation of this pathway as a compensatory response to limit further fat deposition. This is supported by the findings in the present study of a significant negative correlation between SREBP1-C mRNA expression and the relative fat mass in both subcutaneous and retroperitoneal depots in at weaning. It is possible that this effect is mediated in part by leptin, since leptin has been shown to suppress both the transcription and translation of SREBP-1c in adipose tissue in previous studies [312]. However, while plasma leptin levels and leptin mRNA expression were elevated in offspring suckled by cafeteria dams at weaning, there was no significant association between plasma leptin concentrations and SREBP-1c mRNA expression levels at 3 weeks of age in the present study. It therefore appears that other factors related to the increased lipid accumulation in adipose depots, in addition to raised leptin concentrations, also contribute to the suppression of SREBP-1c mRNA expression.

An alternate possibility is that the reduced SREBP-1c expression is a consequence of increased insulin resistance, and accompanying hyperinsulinemia, which is commonly associated with elevated fat mass [476], since SREBP-1c mRNA expression has been shown to be down-regulated by insulin both *in vitro* [477, 478] and *in vivo* [474]. In support of this hypothesis,

in the present study, I observed that the expression of SREBP-1c mRNA in subcutaneous fat mass of males was negatively correlated with the circulating insulin concentrations at weaning.

While the lower mRNA expression of SREBP-1c in the adipocytes in the offspring was unexpected, given that these offspring had a higher relative fat mass and SREBP-1c is considered to be pro-lipogenic, a number of previous studies have suggested that lipogenesis within adipose tissue can be regulated independently of SREBP-1c expression/activation [479]. In one study, targeted disruption of the SREBP-1 gene had a limited effect on the lipogenic gene expression in adipose tissue [480], while other studies have suggested that SREBP-1c mRNA levels are not necessarily correlated with lipogenic gene expression [481, 482]. This, coupled with the lower SREBP-1c mRNA expression in the adipose tissue of offspring suckled by CAF dams despite their higher relative adiposity at weaning, suggests that their increased fat mass is driven by a SREBP-1c-independent mechanism.

While SREBP-1c mRNA levels in male offspring were no longer different between groups at 6 weeks of age, when all pups had been consuming the standard rat chow for 3 weeks, the expression of SREBP-1c mRNA in females was still lower in those offspring suckled by CAF dams. This suggests that the impact of exposure to a cafeteria diet during the major period of adipocyte development in the rodent persists beyond the immediate post-weaning period, even when offspring are consuming a nutritionally-appropriate diet. It is not clear, however, whether these effects on gene expression will persist later in adulthood. In addition, whether the altered SREBP-1c mRNA expression at 6 weeks of age has functional consequences for the lipogeneic capacity of the adipose depots in these offspring remains to be determined.

4.4.1.2. *PPAR-γ mRNA expression*

Whist there was no difference in PPAR-γ expression in retroperitoneal adipose tissue between the groups, PPAR-γ expression in subcutaneous adipose tissue

at 3 weeks of age was significantly higher in the female offspring exposed to a maternal cafeteria diet during the suckling period, and tended to be positively correlated with fat mass.

PPAR-γ is most highly expressed in adipose tissue, where it plays a predominant role in the regulation of adipogenesis and lipogenesis. PPAR-γ also plays a critical role in the regulation of of whole–body lipid metabolism and insulin sensitivity [483-486]. The central role of PPAR-γ in adipocyte differentiation has been firmly established by studies showing that the PPAR-γ null mouse is completely devoid of adipose tissue [487], and that animals born with white adipose tissue knockdown of PPAR-γ developed severe lipodystrophy [488, 489]. Because PPAR-γ has a key role in adipogenesis and lipogenesis, it is possible that increased expression of PPAR-γ observed in the subcutaneous fat mass of female offspring at weaning in the present study might be causally related to the increased fat mass in offspring exposed to the cafeteria diet during the suckling period, particularly since this is the major period of fat deposition in rodents [372]. These findings are consistent with previous studies in sheep which provided evidence that early exposure to an increase in maternal, and hence fetal nutrition, resulted in an upregulation of PPAR-γ in the fetal adipose tissue, which was associated with an increased fat mass in the lambs at the end of the first month of life [375]. Rodent studies have also reported an increased level of PPAR-γ in the white adipose tissue of offspring during the suckling period, when pups are consuming a diet of high-fat milk, compared to when they are weaned onto a relatively low-fat chow diet, and that this was correlated with adipocyte hypertrophy [490]. The observations in the present study therefore suggest that exposure to a maternal cafeteria diet during the suckling period, and thus higher level of fat, results in an upregulation of PPAR-γ mRNA in key adipose depots, which in turn drives increased fat deposition at weaning, at least in female offspring.

While PPAR-γ upregulation may contribute to the increased accumulation of fat mass in female offspring, PPAR-γ mRNA expression was not altered by either the prenatal or early postnatal diet in male offspring, suggesting that the increased fat mass in these offspring is likely to have been programmed via a

PPAR-γ independent mechanism. Differences between the sexes in the impact of maternal high-fat/junk-food feeding on adipose tissue deposition and on adipocyte gene expression is consistent with the results of a previous study [400] which reported that the impact of exposure to a maternal cafeteria diet *in utero* and during the suckling period on body weight, fat deposition and expression of lipogenic genes were more pronounced in female compared to male offspring. This previous study also reported an increase in the expression of PPAR-γ in the adipose tissue of rat female offspring of rat dams fed a junk food diet during pregnancy, lactation and after weaning [400].

One possibility is that the difference in the effect of maternal cafeteria feeding on PPAR-γ expression between male and female offspring was due to interactions between the cafeteria diet and sex hormones, since previous studies have suggested that both estrogen and androgens have a role in regulating PPAR-γ mRNA expression in adipocytes [386, 491]. These studies have suggested that estrogen stimulates PPAR-γ mRNA expression *in vitro* and *in vivo* [492], while androgens decrease it [491, 493]. Differences in lipid metabolism in the fat depots of males and females, which have been well documented in previous studies, may also have contributed to the differential effect of the cafeteria diet during suckling on PPAR-γ mRNA expression in males and females. Previous studies have established that females have higher lipogenic gene expression and higher lipogenic capacity in their fat depots compared to males [494, 495], and this could have contributed to the higher expression of the PPAR-γ gene in females to be increased by exposure to an increased caloric, fat and sugar supply during a critical window of fat development in comparison to males.

Unlike SREBP-1c, the changes in PPAR-γ mRNA expression did not persist at 6 weeks of age, suggesting that the effects of exposure to a cafeteria diet on PPAR-γ mRNA are related to the prevailing nutrient supply, and do not persist when the animals are returned to a nutritionally balanced diet after weaning. Interestingly, PPAR-γ mRNA expression in the retroperitoneal tissue at 6 weeks of age was lower in the female offspring of CAF dams who had been suckled by a control dam compared to all other groups. The reason for this is unclear, however the reduced fat mass in retroperitoneal adipose depot of these

offspring compared to all other groups as reported in Chapter 2 of this thesis, although not statistically significant, could be one reason for the lower PPAR-γ mRNA observed in these offspring at this time point.

4.4.1.3 *G3PDH and FAS mRNA expression*

Previous studies have established that the mRNA expression and enzymatic activity of G3PDH and FAS is increased by signals of positive energy balance, and acts to enhance TG accumulation in adipocytes [496, 497]. Despite the increased fat and sugar intake in the cafeteria dams, and the higher fat mass of all adipose depots in offspring suckled by cafeteria dams at weaning, there was no effect of exposure to the cafeteria diet either before birth or during the suckling period on G3PDH and FAS mRNA expression in either subcutaneous or retroperitoneal adipose tissue at 3 weeks of age. Interestingly, however, G3PDH mRNA levels in the subcutaneous adipose in females and retroperitoneal adipose tissue in both males and females were positively correlated with plasma leptin concentrations at 3 weeks. Similarly, at 6 weeks of age, both G3PDH and FAS mRNA levels in the subcutaneous adipose tissue were also positively correlated with plasma leptin concentrations in males. The observations from the present study therefore suggest that, while G3PDH and FAS mRNA expression appears to be positively related to fat deposition and leptin synthesis, it does not appear to be regulated by diet in either prenatal or early postnatal life. Thus, programming of the transcription of these enzymes is unlikely to be responsible for the increased fat accumulation in offspring suckled by a cafeteria-diet fed dams observed at weaning in the present study.

Interestingly, at 6 weeks of age, G3PDH and FAS mRNA expression was increased in the retroperitoneal adipose tissue of male offspring born to CAF dams who were suckled by a Control dam compared to those pups both born to and suckled by a Control dam. Previous studies have reported that FAS mRNA expression in adipose tissue is acutely sensitive to nutritional and hormonal status [291, 297, 298] and a depressed activity of FAS mRNA has been reported in starved animals which increases to high levels upon re-feeding the animals on

a fat-free diet [299, 300, 303]. Previous studies in rodents have also demonstrated that high-fat feeding decreases FAS mRNA expression [275, 498]. One possible explanation, at least in part, for the increased expression of FAS mRNA in the retroperitoneal adipose tissue of CAF male offspring suckled by a Control dam observed in the present study may be that an exposure to a control diet during the suckling period, following exposure to a cafeteria diet before birth, could have increased the FAS mRNA expression to high levels in these offspring compared to those offspring who were both born to and suckled by a Control dam and that this persisted at 6 weeks of age. Similarly, G3PDH is another lipogenic enzyme [499] whose mRNA expression is influenced by dietary intake [500] and reduced in the adipocytes of obese compared to non-obese individuals [501]. It is therefore possible that exposure to a reduced supply of fat and/or sugar post-weaning, following exposure of the pre-adipocytes to a cafeteria diet before birth, could have resulted in increased G3PDH mRNA expression in CAF offspring suckled by a Control dam compared to those pups both born to and suckled by a Control dam. However it is possible that other factors may also affect G3PDH gene expression in adipocytes and additional studies are required to fully understand the depot and sex specific differences observed.

4.4.1.4 *Adiponectin*

The expression of the insulin-sensitising adipokine, adiponectin, in subcutaneous adipose tissue of females and retroperitoneal adipose tissue of both males and females at weaning were higher in those suckled by CAF dams and were positively correlated to the relative mass of the respective fat depots. This is different to what would be expected, based on findings in adults that adiponectin concentrations are negatively correlated with BMI and fat mass. Indeed this has been cited as one of the reasons for the lower insulin sensitivity in overweight/obese individuals [353, 354, 362]. However, evidence from humans and animals suggests that the relationship between adiponectin concentrations and fat mass may be quite different in infants and young children compared with older children and adults [233, 369, 370, 502]. Human studies

have reported that plasma adiponectin levels are higher in neonates compared to adults, and are positively, rather than negatively, related to neonatal body weight/fat mass [369, 370]. A previous study in sheep also reported an increased expression of adiponectin mRNA in the adipose tissue of fetal sheep which was directly related to the mean fractional growth rate of lambs across the first 30 days of postnatal life [233]. Previous rodent studies also reported that adiponectin inhibits energy expenditure [503, 504] and increases lipid accumulation in adipocytes by suppressing lipolysis and promoting adipocyte differentiation [505, 506]. It has also been reported that overexpression of adiponectin increases fetal fat deposition in mice [502]. This therefore raises the possibility that adiponectin plays an important role in regulating pre- and postnatal growth and fat deposition, and that higher adiponectin secretion may in fact be contributing to the increased fat deposition in offspring suckled by cafeteria-fed dams [369]. Another possible explanation, at least in part, for the increased adiponectin mRNA expression in the adipose tissue of offspring suckled by CAF dam observed in the present study is that activation of PPAR-γ by the cafeteria diet may promote an increase in body fat mass not only by increasing adipocyte differentiation and the number of small adipocytes, but also enhancing adiponectin synthesis in adipose tissue, since adiponectin expression is known to be upregulated by PPAR-γ agonists [507].

Intriguingly, however, at 6 weeks of age adiponectin mRNA concentrations were lower in the retroperitoneal adipose tissue of female offspring born to CAF dam, independent of their dietary exposure during the suckling period. The reasons for this are less clear. One possible explanation for the lower expression of adiponectin in the retroperitoneal adipose tissue of female offspring born to a CAF dam could be a decreased PPAR-γ mRNA expression in the retroperitoneal adipose tissue of these female offspring. However, the lower PPAR-γ mRNA expression can only partially explain this observation, since PPAR-γ mRNA expression was observed only in those offspring born to CAF dam who were suckled by a Control dam. It is also possible that adiponectin mRNA expression could be programmed before birth by effects on pre-adipocytes. However, further studies are needed to investigate this further.

The fact that the effects on adiponectin mRNA were only observed in female offspring provides further evidence of sexual dimorphism in the effects of early life exposure to a cafeteria diet on adipocyte gene expression. Sexual dimorphism in adiponectin mRNA expression and circulating adiponectin levels has been reported previously in both rodents and humans [508-510]. Although these studies reported sex differences in the expression of adiponectin, they also concluded that the regulation of adiponectin expression and secretion is complex and appears to be dependent on other factors in addition to gonadal steroids. However, sexual dimorphism in the expression of adiponectin mRNA in response to increased maternal nutrition has not been previously described. Although previous studies have reported that adiponectin levels are associated with insulin sensitivity [358, 511], no studies have determined whether this association is the same between the sexes. As there were no differences in insulin sensitivity between treatment groups and between sexes in the present study, it does not appear that the changes in adiponectin mRNA expression observed in this study had any impact on insulin sensitivity. Thus, while the results of the present study suggest that early overnutrition may have long-term consequences for the function of adipose tissue in later life, the mechanisms underlying the sex specific differences in the effect of prenatal/early postnatal exposure to the cafeteria diet on adiponectin expression require further investigation.

4.4.1.5 *Leptin*

In the present study, exposure to the maternal cafeteria diet during the suckling period was associated with a significant increase in the expression of leptin mRNA in both subcutaneous and retroperitoneal adipose tissue of both male and female offspring. This increase in leptin mRNA expression appeared to be a consequence of increased accumulation of lipid, since there was also a direct relationship between leptin mRNA expression in both adipose fat depots and relative fat mass of respective fat depot. No changes in leptin mRNA expression were observed between groups or between sex in the subcutaneous adipose tissue at 6 weeks of age, when offspring had been consuming the standard

rodent diet for 3 weeks and there were no longer any differences in fat mass between the treatment groups. However, similar to the expression of adiponectin, expression of leptin mRNA in the retroperitoneal adipose tissue was decreased in female offspring exposed to the cafeteria diet before birth, and was positively correlated with relative fat mass.

It has been demonstrated in both humans and animal models that chronic exposure to elevated leptin levels leads to the development of a resistance to its actions [332, 336, 512]. The resulting leptin resistance has been suggested to be an important factor which contributes both to the development of human obesity [336] and to the programming of the obese phenotype by specific nutrient exposures *in utero* [106]. Previous studies in both humans [337, 338, 340] and sheep [339, 341, 342] have reported that leptin has a potential role as a signal of fat stores before birth; in both humans [340] and sheep [341, 342] changes in fetal plasma leptin concentrations occur in parallel with changes in intrauterine fat deposition and there is a positive correlation between leptin concentrations in the umbilical cord blood at delivery and anthropometric measures of neonatal adiposity in human infants [337, 338]. It has also been reported that leptin is secreted into the circulation in proportion to the total body fat mass, and therefore acts as a circulating signal of body fat stores [323-325]. A nutritional influence on the ontogenic profile of leptin in the neonatal circulation has also been reported in lambs [347]. In agreement with previous studies [513], the data from the present study therefore suggests that increased maternal nutrition during suckling period increased the leptin gene expression which enhanced leptin production by fat cells, thus raising the circulating leptin levels. It is possible that this may program leptin resistance in later life, but this remains to be directly demonstrated.

4.4.2 Differential expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal adipose tissue in the male and female offspring at 3 weeks and 6 weeks of age

I found depot specific differences in the mRNA expression of key adipogenic and lipogenic genes in subcutaneous and retroperitoneal adipose tissue which were present independent of treatment group. At 3 weeks of age, SREBP-1c mRNA was expressed more highly in the subcutaneous depot, while FAS mRNA was more highly expressed in the retroperitoneal depot in both males and females. In females, there were also differences between the depots in the mRNA expression of PPAR-γ, G3PDH, adiponectin and leptin mRNA, which were all more highly expressed in subcutaneous compared to visceral adipose tissue, but these differences were not present in males.

Differences between subcutaneous and visceral fat depots in the expression of key adipogenic and lipogenic transcription factors have been widely reported in previous studies [514-516]. Furthermore, this is thought to be the major factor driving differences in the properties of these respective fat depots, and therefore the different metabolic consequences of visceral vs subcutaneous obesity [517, 518]. The pattern of expression of SREBP-1c in visceral and subcutaneous fat in the 3 week old offspring in the present study is in agreement with previous human studies which also reported a markedly lower expression of SREBP-1c in visceral adipose tissue (omental fat) compared to subcutaneous adipose tissue in both males and females [472]. The potential mechanisms which have been suggested to explain these differences in previous studies are the higher lipolytic capacity of metabolically more active visceral adipose tissue [519] and the lower insulin responsiveness of visceral adipose depot compared to subcutaneous adipose tissue [520].

The increased expression of lipogenic genes including G3PDH, FAS, PPAR-γ, adiponectin and leptin in subcutaneous compared to visceral fat have also been reported in a number of previous studies in humans and rodents [521-524]. This is thought to be related to the role of subcutaneous fat as the primary depot for

fat storage, at least in metabolically healthy individuals, and the increased size of fat cells in this depot compared to visceral depots [521-524]. In the present study, I observed that both males and females deposited more subcutaneous adipose tissue compared to retroperitoneal adipose and subcutaneous adipose tissue mass was 3-10 fold higher than retroperitoneal adipose tissue in all offspring at 3 weeks of age, independent of treatment group (Chapter 2). Given that the mRNA expression of key adipogenic and lipogenic genes in the present study (SREBP-1c, adiponectin and leptin) were positively correlated to the relative mass of the respective fat depots, it is tempting to speculate that these alterations in the expression of key adipogenic and lipogenic genes could be related to the differences in the mass of these fat depots. However, with the exception of FAS, only females exhibited depot specific expression of adipogenic and lipogenic genes at this time point. There is evidence that female adipocytes have increased lipogenic capacity and lipid synthesis in subcutaneous adipose depots compared to males [525]. Thus, the fact that differential expression of these genes between depots was seen in females, but not males, may be due to the higher lipid content and lipogenic capacity of subcutaneous fat in females.

Interestingly, the pattern of gene expression between the fat depots at 6 weeks was in stark contrast to those at weaning and the expression of PPAR-γ, G3PDH and FAS was higher in the retroperitoneal adipose tissue compared to subcutaneous adipose tissue in both males and females. In females, the mRNA expression of adiponectin and leptin mRNA were also more highly expressed in retroperitoneal fat depot compared to subcutaneous adipose tissue, which was in contrast to the pattern at 3 weeks. SREBP-1c expression, however, was still expressed more highly in the subcutaneous adipose tissue compared to retroperitoneal adipose tissue in females at this age. One possible explanation for these observations is that, in the present study, the mass of retroperitoneal adipose tissue relative to body weight was increased in both males and females between weaning to 6 weeks of age, whilst subcutaneous adipose tissue mass relative to body weight was either decreased or unchanged, independent of treatment group (Chapter 2). This suggests that there would have been higher

rates of lipogenesis in retroperitoneal fat compared to subcutaneous adipose tissue during this time in both male and female offspring. This possibly accounts, at least in part, for the increased expression of key adipogenic and lipogenic genes in the retroperitoneal adipose tissue in the offspring at 6 weeks of age.

A previous study [526] reported a sharp decrease in the fractional cell proliferation rate in subcutaneous, inguinal and retroperitoneal fat pad at the end of the suckling period in rats which remained nearly constant throughout puberty, but this study did not specifically compare the rates in the subcutaneous and retroperitoneal fat depots. Although I did not directly measure adipocyte proliferation rate in the present study, the fact that the subcutaneous adipose tissue weight relative to body weight was decreased or unchanged between weaning to 6 weeks of age suggests a decrease in the cell proliferation rate and/or cellular hypertrophy in this fat depot; which would be consistent with the lower expression of key adipogenic and lipogenic genes in this fat depot compared to retroperitoneal adipose tissue in the offspring at 6 weeks. These patterns of gene expression between depots were, however, different to those seen in mature rodents, suggesting that the adipose depots are not fully mature at this time [527]. Although sex and depot specific differences in the intrinsic properties of adipocytes are well documented, the mechanisms leading to these differences are less clear and further investigation is needed.

4.5. SUMMARY

In summary, the results of this Chapter have demonstrated that exposure to increased maternal nutrition during the early postnatal period (and to some extend the prenatal period) results in altered expression of key adipogenic and lipogenic genes, particularly in the subcutaneous adipose depot. I have reported sex and depot specific differences in the expression of key adipogenic and lipogenic transcription factors in the adipose tissue of rat offspring exposed to cafeteria diet during the suckling period, with more prominent effects in females. I have presented evidence that these differences were correlated with changes in adiposity and circulating levels of plasma hormones. I have also shown that

an increase in nutrient supply during the early postnatal period is more important than the prenatal period in determining fat deposition, and that increased fat deposition observed in the offspring exposed to maternal cafeteria diet during the suckling period may be due, at least in part, to increased adipogenic and lipogenic gene expression in adipose tissue. However, the majority of these effects did not persist after offspring are weaned to a control diet suggesting that they do not contribute to long-term programming of fat deposition, at least as long as rats are maintained on a nutritionally balanced chow diet. The findings from the present study also suggest that key adipogenic and lipogenic transcription factors, in particular PPAR-γ and SREBP-1c, are important in the differential sensitivity of subcutaneous and retroperitoneal fat depots to maternal overnutrition during the suckling period and in determining sex differences in response to maternal cafeteria diet feeding.

CHAPTER 5

CHAPTER 5: EXPOSURE TO MATERNAL HIGH-FAT AND HIGH-SUGAR CAFETERIA DIET DURING LACTATION INCREASES OFFSPRING SUSCEPTIBILITY TO DIET-INDUCED OBESITY

5.1 INTRODUCTION

As highlighted in the preceding Chapters, a large number of studies have suggested that exposure to maternal overnutrition, in particular excess intakes of fat and/or sugar, during the perinatal period is associated with an increased risk of obesity and poor metabolic health outcomes in the offspring [93, 107, 117, 394-396, 400, 524]. More recently, there has been growing interest in determining the separate contributions of exposure to high fat/high sugar diets *in utero* and in the early postnatal period on the short/ and longer term metabolic health outcomes of the offspring. Indeed, a number of studies have reported distinct effects of exposures during these two periods for the long-term outcomes in the offspring [102, 103, 107, 108]. While the majority of studies of maternal obesity/maternal high-fat feeding have reported higher body weights and fat mass in the offspring independent of the diet after weaning, it is also clear that these effects are greatly exaggerated when offspring are weaned onto a high-fat and/or high-sugar diet [93, 117, 400]. However, the relative contribution of exposure to a high-fat/high-sugar diet before birth and during early postnatal life to this increased susceptibility to diet-induced obesity in the offspring is unknown.

In Chapter 2 of this thesis, I demonstrated that offspring who were suckled by a dam consuming a cafeteria diet had a significantly higher relative fat mass at weaning compared to those suckled by control dams, independent of the nutritional environment they were exposed to before birth. Conversely, fat mass in offspring born to cafeteria-fed dams suckled by a Control dam was not different from offspring who were exposed to the control diet during both the prenatal and postnatal periods. These data strongly suggest that the nutritional

environment during the suckling period is more important for the deposition of fat during the suckling period than exposure before birth. Whilst relative fat mass was no longer different between groups at 6 weeks of age, after all offspring had been consuming a standard nutritionally balanced chow since weaning, I identified several differences between groups in adipocyte gene expression at 6 weeks of age, suggesting that the potential of these cells to store lipid may be persistently altered by exposure to the cafeteria diet during the perinatal period.

The aim of the present study was to determine the effect of exposure to a high-fat, high-sugar cafeteria diet before birth and/or during suckling period on susceptibility to diet-induced obesity in the offspring in young adulthood. This study also aimed to determine the impact of this secondary exposure to the cafeteria on adipocyte gene expression in the offspring. A secondary aim was to establish if the effects of perinatal exposure to the cafeteria diet on these outcomes differed between male and female offspring.

5.2 MATERIALS AND METHODS

5.2.1 Animals and feeding regime

All procedures were approved by the Adelaide University Animal Ethics Committee. The same procedures were followed as described in Chapter 2.

5.2.2 Mating and pregnancy

The same procedures were followed as described in Chapter 2.

5.2.3 Cross-fostering

Cross-fostering procedures were performed as described in Chapter 2.

5.2.4 Offspring feeding regime

After weaning, all offspring were provided with *ad libitum* access to the standard chow diet, and remained on the diet for 7 weeks post weaning. All offspring were housed with a same-sex litter mate with two animals in each cage during this time. After 10 weeks of age, all offspring were given free access to both the standard rat chow and cafeteria diet in order to determine their relative preference for the palatable diet and susceptibility to diet induced weight gain/fat deposition (Figure 5.1). Food intake was measured every 2 days by subtracting the amount left uneaten in the cage from the amount initially provided. The total intake of each food type was recorded and macronutrient intake for each cage determined based on the nutritional composition of the food consumed (as provided by the manufacturer). Food intake was divided by the number of offspring in the cage to determine individual offspring food intake. Pups were weighed once per week from 3 weeks of age until the end of the experiment.

5.2.5 Post-mortem and tissue collection

At 3 months of age, 3 weeks after the start of the food preference diet, one male and one female pup from each litter were killed for the determination of body fat mass. Post-mortems were carried out as described in Chapter 2. The same procedures for euthanasia and collection and storage of tissues were used as previously described in Chapter 2. Samples of retroperitoneal (visceral fat) and subcutaneous fat were snap frozen in liquid nitrogen and stored at -80^{0}C for subsequent molecular analyses.

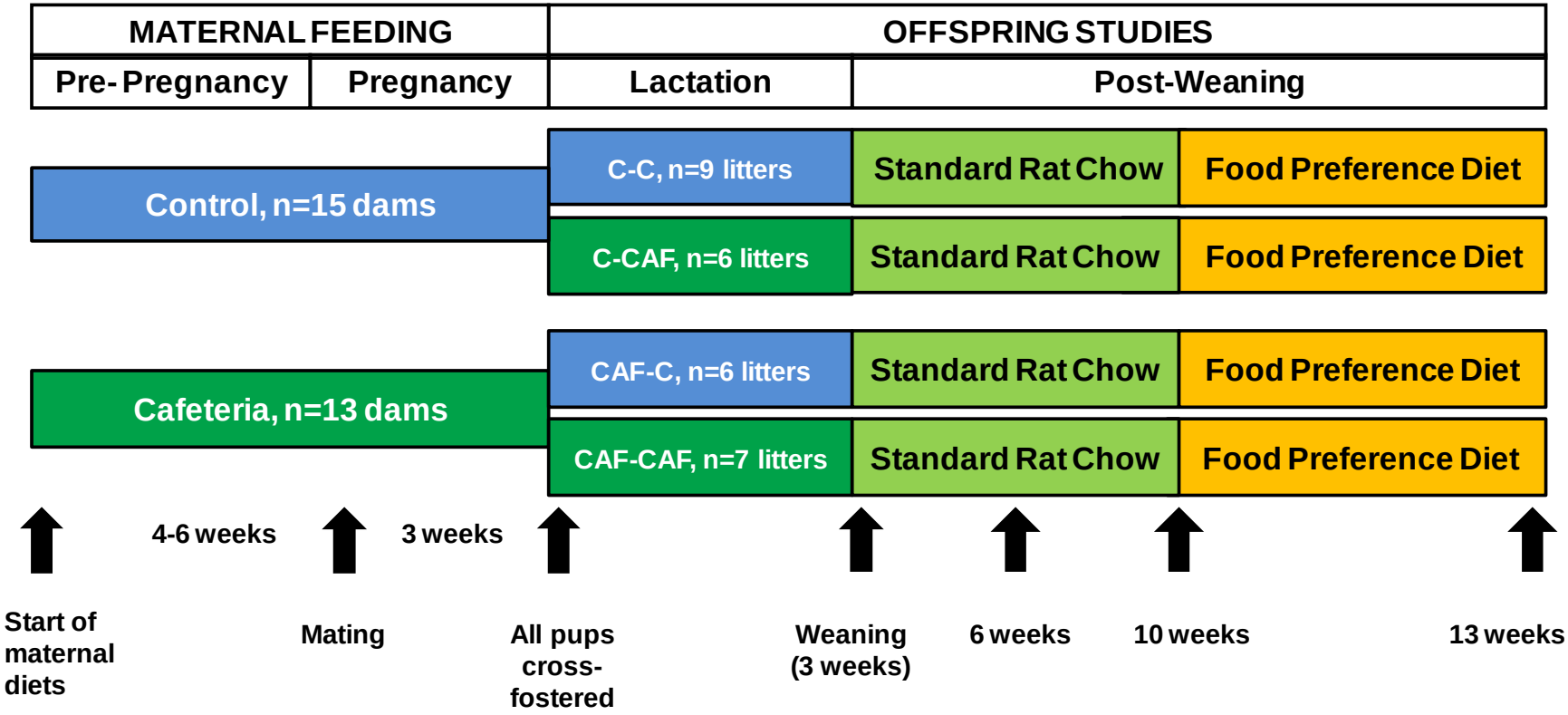


Figure 5.1 Experimental design: Offspring of Control (n=15 litters) and CAF dams (n=13 litters) were cross-fostered to another dam which gave birth within the same 24 hour period from either the same or different dietary treatment group. Offspring were kept with their foster mother until weaning (3 weeks of age), and then provided *ad libitum* access to the standard chow diet for 7 weeks post weaning. After 10 weeks of age, all offspring were given free access to both the standard rat chow and cafeteria diet until the end of the experiment for the determination of food preferences.

5.2.6 Determination of plasma glucose, NEFA, insulin and leptin concentrations

As described in Chapter 2, plasma concentrations of glucose and NEFA were determined using the Infinity Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA, USA) and the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan), respectively. Assays were conducted using Konelab 20 (Thermo Scientific, Vantaa, Finland). Plasma insulin and leptin concentrations at 3 months were measured by immunoassay using the ALPCO Insulin (Rat) Ultrasensitive ELISA kit (ALPCO Diagnostics, Salem, NH, USA) and the Crystal Chem Rat Leptin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA). All assays were conducted according to manufacturer's instructions and the intra- and inter-assay coefficients of variation(CoV) for glucose and NEFA assays were < 5% and the intra- and inter-assay coefficients of variation(CoV) for insulin and leptin assays were <10%.

5.2.7 RNA extraction and reverse transcription

Procedures for RNA extraction and reverse transcription were carried out as described in Chapter 4.

5.2.8 Determination of gene expression in the subcutaneous and retroperitoneal adipose tissue

Procedures for Real Time PCR were carried out as described in Chapter 4.

5.2.9 Statistical analyses

Data are presented as mean ± SEM. The dam was used as the unit of analysis. Effects of maternal cafeteria diet during pregnancy and/or lactation on offspring body weight were determined using a repeated measures ANOVA. The effect of

prenatal vs postnatal nutritional environment and their interaction on offspring body fat mass, plasma hormones and metabolite concentrations and gene expression in the subcutaneous and retroperitoneal tissues at 3 months were determined using three-way ANOVA, with sex, prenatal and postnatal diet as factors. As three-way ANOVA revealed many differences between the sexes in both mean values and variation within groups, the effect of prenatal vs postnatal environment and their interaction on offspring body fat mass, plasma hormone and metabolite concentrations and gene expression in the subcutaneous and retroperitoneal tissues were determined separately in male and female offspring using a 2 way ANOVA. Where significant interactions were identified by ANOVA, the effect of the prenatal and postnatal exposure within each sex were analysed separately by a Student's T-Test. Relationships between gene expression in subcutaneous and retroperitoneal fat and mass of the respective fat depot in the male and female offspring at 3 months were determined by simple linear regression analysis. Relationships between relative total fat mass, fat mass of individual fat depots and plasma hormone and metabolite concentrations in the male and female offspring at 3 months and relationships between gene expressions in subcutaneous and retroperitoneal fat and plasma hormone and metabolite concentrations in the male and female offspring at 3 months were similarly determined. The expression of adipogenic and lipogenic genes between the subcutaneous and retropeitoneal fat in the male and female offspring at 3 months were analysed by Student's T-Test. The repeated measure ANOVAs were performed using Stata 10 (Stata Corp LP, Texas, USA). All other analyses were performed using SPSS for Windows Version 17.0 (SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered statistically significant in all analyses. A Grubb's test was performed to identify significant outliers in each group prior to commencing the analysis.

5.3 RESULTS

5.3.1 Offspring growth during the control diet period and food preference diet period

In males, there was an interaction between prenatal and postnatal dietary exposure on offspring body weight during the control diet period post weaning, such that offspring exposed to the cafeteria diet at any stage during the perinatal period were lighter than those exposed to the control diet both before birth and during the suckling period (Figure 5.2 A). There were, however, no differences in the body weight of male offspring between the groups during the period when they had access to both the control and cafeteria diets.

In females, there was no difference in the offspring body weight between the groups during the post weaning control diet period. During the period when offspring had access to both the control and cafeteria diet, however, female offspring born to CAF dams which were suckled by a Control dam were lighter than offspring in all other treatment groups (Figure 5.2 B).

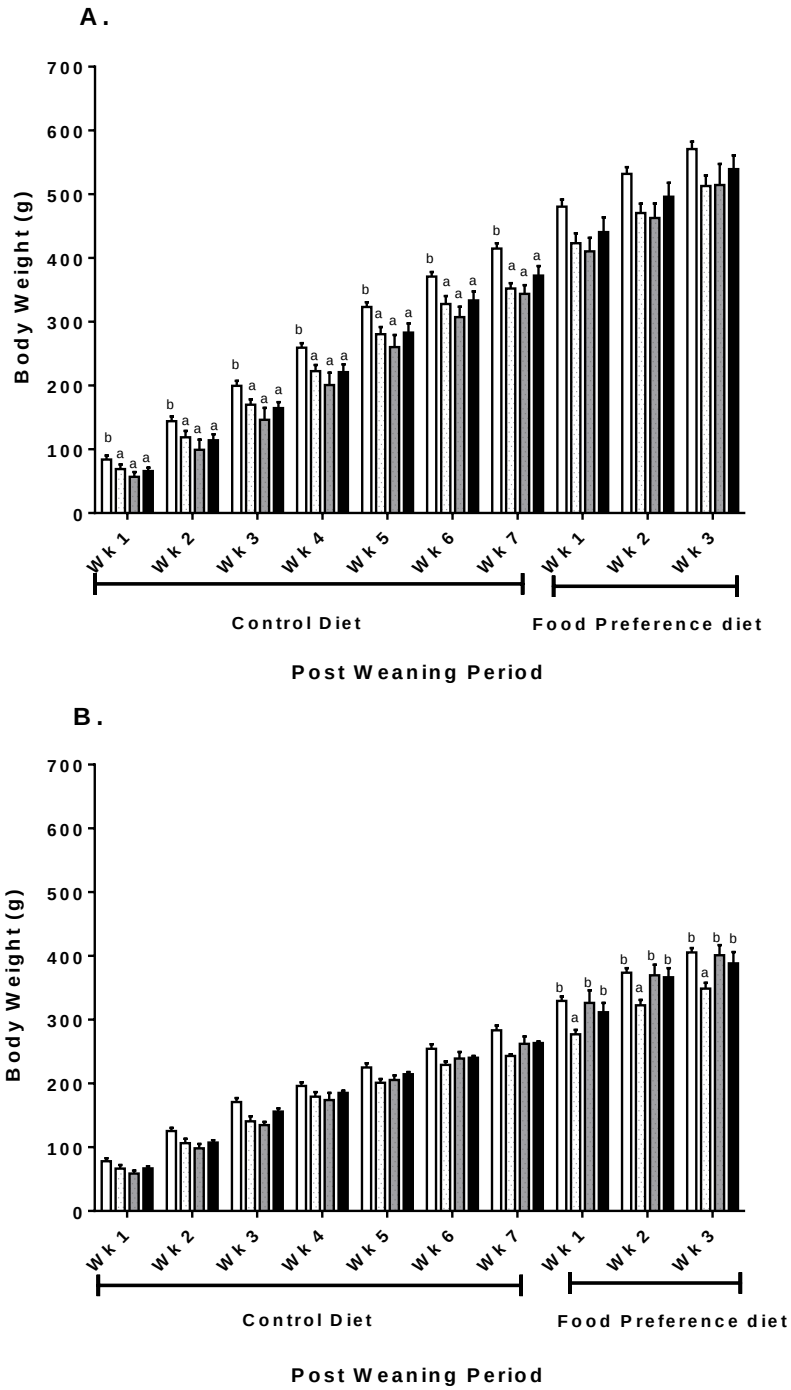


Figure 5.2 Body weight of male (A) and female (B) offspring during the post weaning control diet period and food preference diet period in offspring of C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups at 3 months of age. Values are expressed as mean ± SEM. Different superscripts denote significant differences between mean values (*P<0.05)*.

5.3.2 The effect of prenatal and postnatal diet on offspring body fat mass at 3 months of age

At 3 months of age, after 3 weeks on the cafeteria diet, there were no differences between the groups in the total body fat mass and fat mass in the individual fat depots in male offspring (Figure 5.3 A, Figure 5.4, Table 5.1). In females, however, those offspring who had been exposed to the cafeteria diet during the suckling period had significantly higher total body fat mass, omental and gonadal fat mass as a percentage of body weight, independent of dietary exposure before birth (Figure 5.3 B, Table 5.1). Intriguingly, at 3 months, relative subcutaneous fat mass was actually significantly reduced in those female offspring who had been exposed to cafeteria diet before birth, independent of dietary exposure during the suckling period (Figure 5.4). There were no differences between the groups in the relative mass of the retroperitoneal or interscapular fat depots after 3 weeks on the cafeteria diet in female offspring (Figure 5.4, Table 5.1).

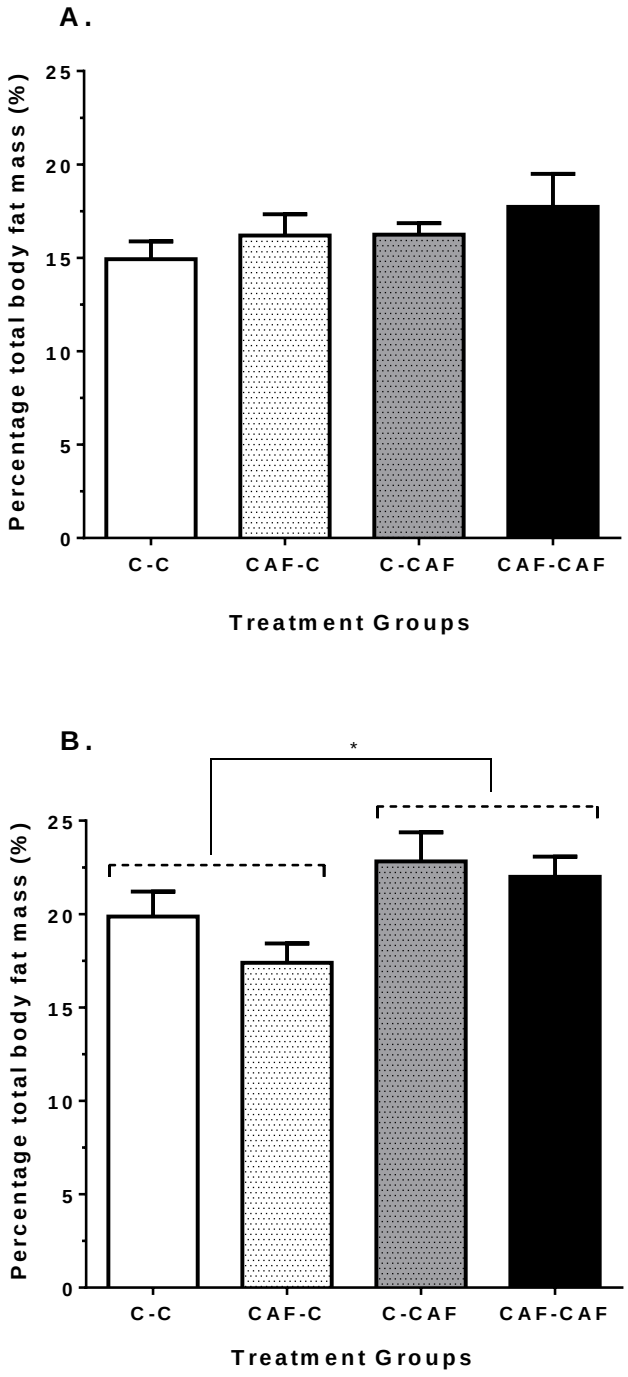


Figure 5.3 Total body fat mass (expressed as a percentage of body weight) in male (A) and female (B) offspring in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

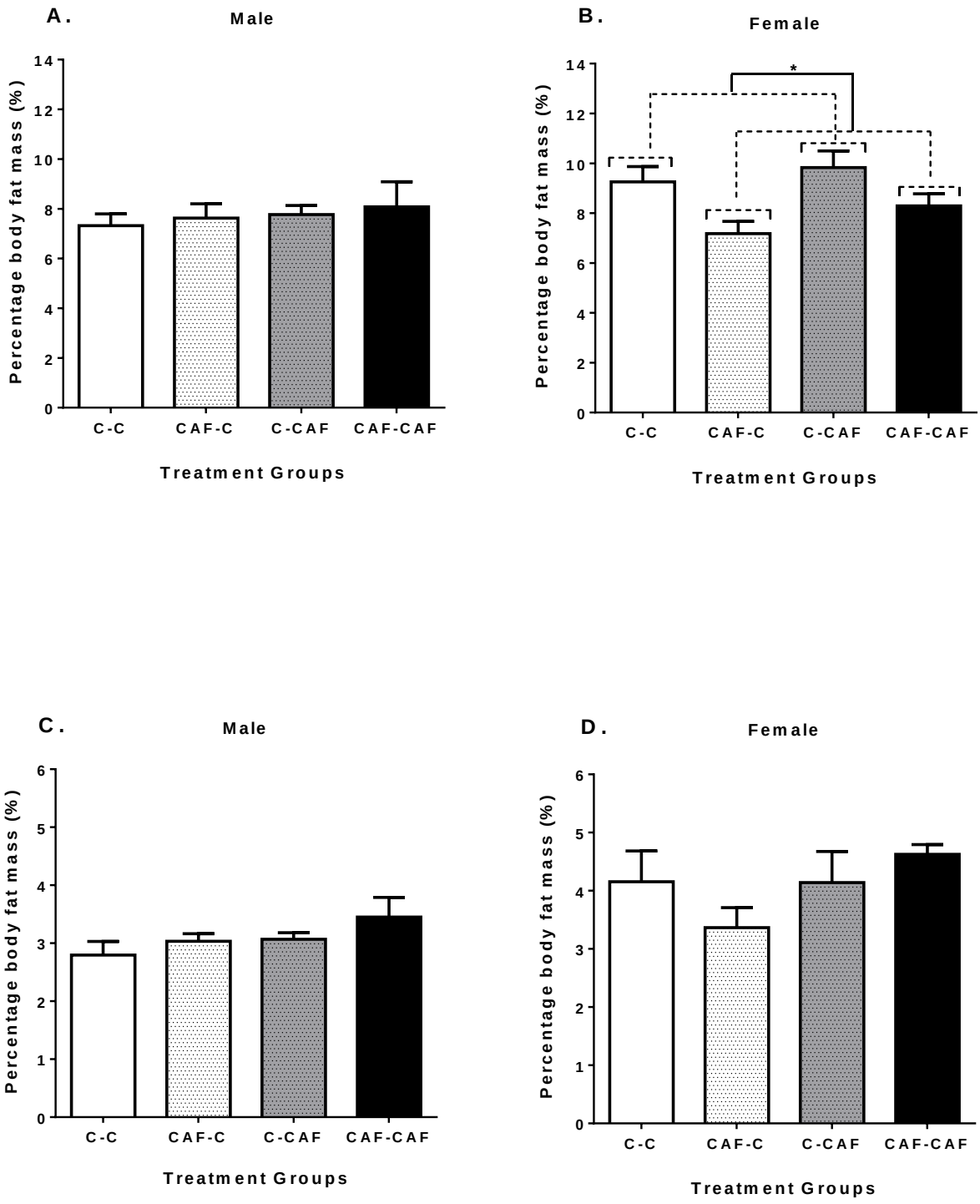


Figure 5.4 Subcutaneous (A, B) and retroperitoneal fat mass (C, D) (expressed as a percentage of body weight) in male (A, C) and female (B, D) offspring in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

Table 5.1 Mass of individual fat depots expressed as a percentage of body weight in male and female offspring in the C-C, CAF-C, C-CAF and CAF-CAF groups at 3 months of age. Values are expressed as means ± SEM. * denotes significance at *P<0.05*.

	Male				Female			
	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Gonadal fat	2.41 ± 0.19	2.86 ± 0.32	2.60 ± 0.14	3.22 ± 0.30	3.20 ± 0.42	3.96 ± 0.16	4.74 ± 0.37*	5.19 ± 0.51*
Interscapular fat	0.34 ± 0.04	0.43 ± 0.04	0.45 ± 0.04	0.45 ± 0.02	0.56 ± 0.08	0.45 ± 0.07	0.54 ± 0.13	0.59 ± 0.09
Omental fat	2.06 ± 0.15	2.26 ± 0.18	2.36 ± 0.18	2.53 ± 0.20	2.71 ± 0.25	2.44 ±0.12	3.57 ± 0.31*	3.30 ± 0.16*

5.3.3 Effect of prenatal and postnatal maternal diet on plasma hormones and metabolite concentrations at 3 months of age

Exposure to the cafeteria diet either before birth or during the suckling period had no effect on plasma concentrations of glucose, NEFA, Insulin and leptin in the male offspring after 3 weeks on the cafeteria diet (Table 5.2).

In females, however, those offspring suckled by CAF dams had significantly higher plasma leptin concentrations at 3 months of age independent of dietary exposure before birth, consistent with the increased percentage body fat in these offspring (Table 5.2). Those female offspring who were exposed to the cafeteria diet before birth, however, exhibited higher plasma insulin and reduced plasma NEFA concentrations at 3 months of age, independent of the dietary exposure during the suckling period (Table 5.2).

5.3.4 The relationship between percentage fat mass and plasma glucose, NEFA, insulin and leptin concentrations at 3 months of age

Although the maternal diet during suckling period had no effect on the plasma hormone and metabolite concentrations in the male offspring at 3 months, plasma leptin concentrations were positively correlated with percentage total body fat mass and the relative mass of gonadal fat, retroperitoneal fat, omental fat and subcutaneous fat in the male offspring at 3 months of age (Table 5.3). A significant positive correlation between plasma leptin concentrations and percentage total body fat mass and mass of gonadal fat, retroperitoneal fat, omental fat and subcutaneous fat was also present in female offspring (Table 5.3).

In male offspring, there were also significant positive correlations between plasma glucose concentrations and percentage total body fat mass and mass of gonadal fat, omental fat and subcutaneous fat when the data from all animals

were combined (Table 5.3). Plasma glucose concentrations also tended to be positively correlated with the percentage total body fat (*P=0.07*) and omental fat (*P=0.06*), and plasma NEFA concentrations tended to be positively correlated with relative subcutaneous (*P=0.05*) and interscapular (*P=0.06*) fat mass in the female offspring at 3 months of age (Table 5.3). No correlations were observed between plasma concentrations of insulin and percentage total body fat or the relative mass of individual fat depots at 3 months of age in either males or females (Table 5.3).

Table 5.2 Plasma concentrations of glucose, NEFA, insulin and leptin in male and female offspring in the C-C, C-CAF, CAF-C and CAF-CAF groups at 3 months of age. Values are expressed as means ± SEM. * denotes significance at *P<0.05.*

Parameter	Male				Female			
	C-C	CAF-C	C-CAF	CAF-CAF	C-C	CAF-C	C-CAF	CAF-CAF
Glucose (mmol/L)	20.46 ± 1.44	21.92 ± 1.57	20.91 ± 1.96	25.27 ± 2.46	18.54 ± 1.20	15.48 ± 0.74	20.33 ± 1.36	20.10 ± 3.10
NEFA(μEq/L)	0.65 ± 0.12	0.87 ± 0.22	0.84 ± 0.12	0.37 ± 0.06	0.63 ± 0.05	0.40 ± 0.10*	0.64 ± 0.09	0.41 ± 0.07*
Insulin (ng/ml)	2.24 ± 0.81	1.08 ± 0.40	1.06 ± 0.67	3.52 ± 0.89	1.56 ± 0.44	2.16 ± 0.48*	1.10 ± 0.56	3.16 ± 0.64*
Leptin (ng/ml)	31.22 ± 2.05	31.07 ± 2.12	28.76 ± 2.34	34.74 ± 5.42	29.91 ± 1.68	23.54 ± 3.58	37.29 ± 6.24*	31.72 ± 2.67*

Table 5.3 The relationship between percentage total body fat mass and percentage individual fat mass with plasma glucose, NEFA, insulin and leptin concentrations, independent of treatment groups, in the male and female offspring at 3 months of age.

	Glucose		NEFA		Insulin		Leptin	
	Male	Female	Male	Female	Male	Female	Male	Female
Total body fat mass	r^2 = 0.499, *P< 0.001*	r^2 = 0.146, *P= 0.07*	ns	ns	ns	ns	r^2 = 0.503, *P< 0.001*	r^2 = 0.496, *P< 0.001*
Gonadal fat	r^2 = 0.451, *P< 0.001*	ns	ns	ns	ns	ns	r^2 = 0.338, *P< 0.01*	r^2 = 0.199, *P< 0.05*
Interscapular fat	ns	ns	ns	r^2 = 0.156, *P= 0.06*	ns	ns	ns	ns
Retroperitoneal fat	ns	ns	ns	ns	ns	ns	r^2 = 0.203, *P< 0.05*	r^2 = 0.410, *P< 0.01*
Omental fat	r^2 = 0.664, *P< 0.001*	r^2 = 0.152, *P= 0.06*	ns	ns	ns	ns	r^2 = 0.384, *P< 0.01*	r^2 = 0.331, *P< 0.01*
Subcutaneous fat	r^2 = 0.367, *P< 0.01*	ns	ns	r^2 = 0.168, *P= 0.05*	ns	ns	r^2 = 0.470, *P< 0.001*	r^2 = 0.403, *P< 0.01*

5.3.5 Effect of prenatal and postnatal nutrition on the expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal tissue of male and female offspring at 3 months of age

5.3.5.1 SREBP-1c mRNA expression

After all offspring had free access to the cafeteria diet for 3 weeks, the relative expression of SREBP-1c mRNA in subcutaneous adipose tissue was higher in those male offspring who had been exposed to the cafeteria diet before birth, independent of dietary exposure during the suckling period (Figure 5.5 A). There was no difference between the groups in the expression of SREBP-1c mRNA in the subcutaneous adipose tissue in females (Figure 5.5 B) and no differences between the groups in the expression of SREBP-1c mRNA in retroperitoneal tissue in either male or female offspring (Figure 5.5 C, D).

There was no relationship between the expression of SREBP-1c mRNA in subcutaneous or retroperitoneal fat and the relative mass of the respective fat depot in either male or female offspring. However, the expression of SREBP-1c mRNA in subcutaneous tissue was positively related to circulating insulin concentrations in males and circulating NEFA concentrations in females (Table 5.4). The expression of SREBP-1c mRNA in retroperitoneal adipose tissue was negatively correlated with circulating NEFA concentrations in males, but not in females. There was no significant correlation between the expression of SREBP-1c mRNA in either subcutaneous or retroperitoneal fat and plasma glucose or leptin concentrations in either male or female offspring (Table 5.4).

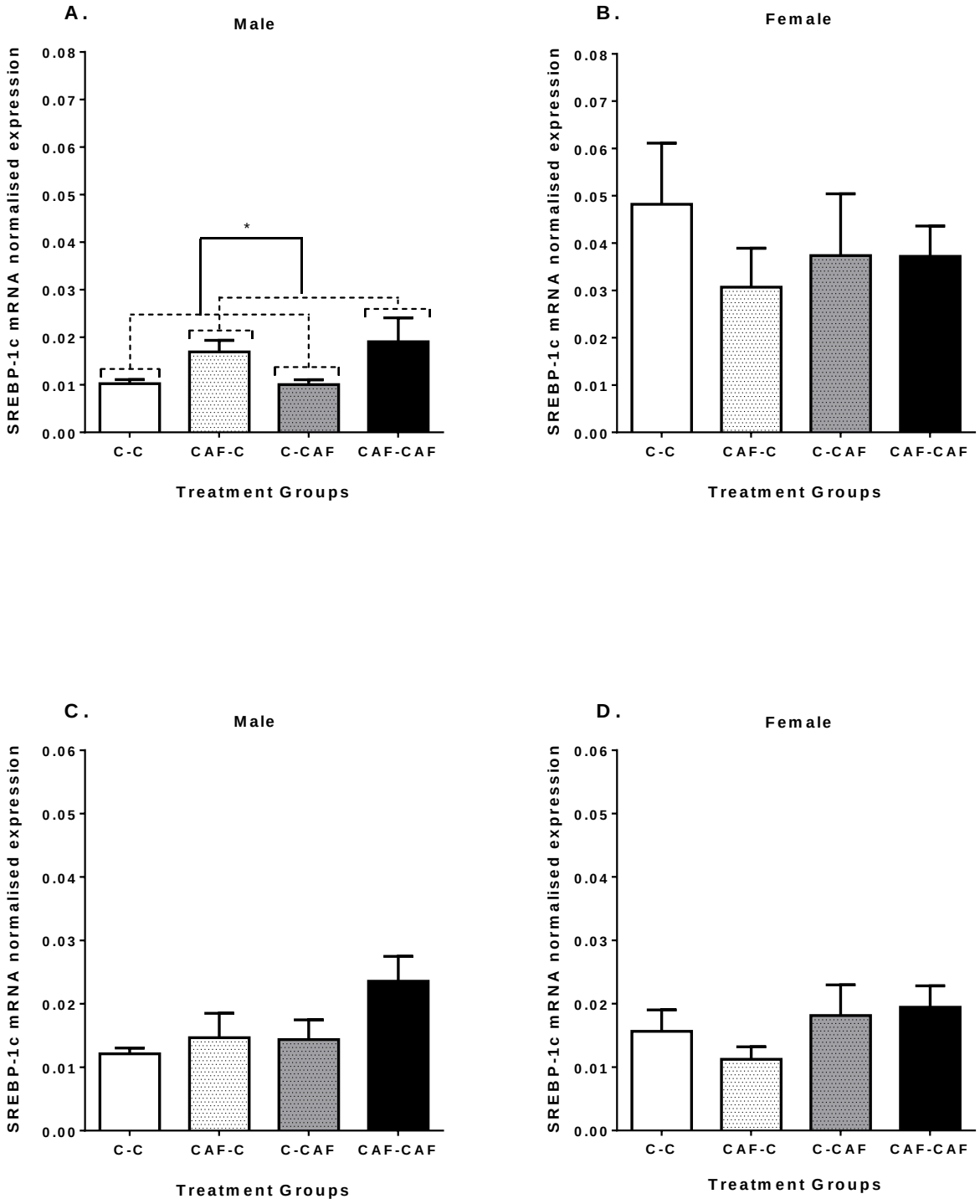


Figure 5.5 The relative expression of SREBP-1c mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

Table 5.4 The relationship between the normalised expression of adipogenic and lipogenic genes in the subcutaneous and retroperitoneal adipose tissues and plasma glucose, NEFA, insulin and leptin concentrations, independent of treatment groups, in male and female offspring at 3 months of age.

Subcutaneous adipose tissue	Glucose		NEFA		Insulin		Leptin	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
SREBP-1c	ns	ns	ns	r^2 = 0.15, *P= 0.06*	r^2 = 0.22, *P< 0.05*	ns	ns	ns
PPAR-γ	ns	ns	ns	r^2 = -0.15, *P= 0.07*	ns	ns	ns	ns
G3PDH	ns	ns	ns	ns	ns	ns	ns	ns
FAS	ns	ns	ns	ns	r^2 = 0.18, *P< 0.05*	r^2 = 0.16, *P= 0.07*	ns	ns
Adiponectin	ns	ns	ns	r^2 = -0.25, *P< 0.05*	ns	ns	ns	ns
Leptin	ns	ns	ns	ns	ns	ns	r^2 = 0.30, *P< 0.01*	ns
Retroperitoneal adipose tissue								
SREBP-1c	ns	ns	r^2 = -0.16, *P= 0.06*	ns	ns	ns	ns	ns
PPAR-γ	ns	ns	ns	ns	ns	ns	ns	ns
G3PDH	ns	ns	r^2 = -0.16, *P= 0.05*	ns	ns	ns	ns	ns
FAS	ns	ns	r^2 = -0.19, *P< 0.05*	ns	ns	ns	ns	ns
Adiponectin	ns	ns	ns	ns	ns	ns	ns	ns
Leptin	ns	ns	ns	ns	ns	ns	r^2 = 0.29, *P< 0.01*	r^2 = 0.19, *P< 0.0*

5.3.5.2 PPAR-γ mRNA expression

There was no effect of either prenatal or postnatal exposure to the cafeteria diet on PPAR-γ mRNA expression in subcutaneous or retroperitoneal adipose tissue after 3 weeks of access to the cafeteria diet in either males or in females (Figure 5.6 A, B, C, D). There was, however, a significant positive relationship between the expression of PPAR-γ mRNA in subcutaneous fat and relative subcutaneous fat mass in males, but not females, at this time (Figure 5.7). There were no significant relationships between PPAR-γ mRNA expression in retroperitoneal fat and the relative mass of this fat depot in either males or females at 3 months of age.

PPAR-γ mRNA expression in either the subcutaneous or retroperitoneal adipose tissue was not related to plasma concentrations of glucose, insulin or leptin in either male or female offspring at 3 months of age (Table 5.4). However, the expression of PPAR-γ mRNA in the subcutaneous adipose tissue tended (*P=0.07*) to be negatively correlated with plasma NEFA concentrations in female offspring (Table 5.4). There was no relationship between the expression of PPAR-γ mRNA in the retroperitoneal adipose tissue and plasma concentrations of NEFA in either male or female offspring at 3 months of age (Table 5.4).

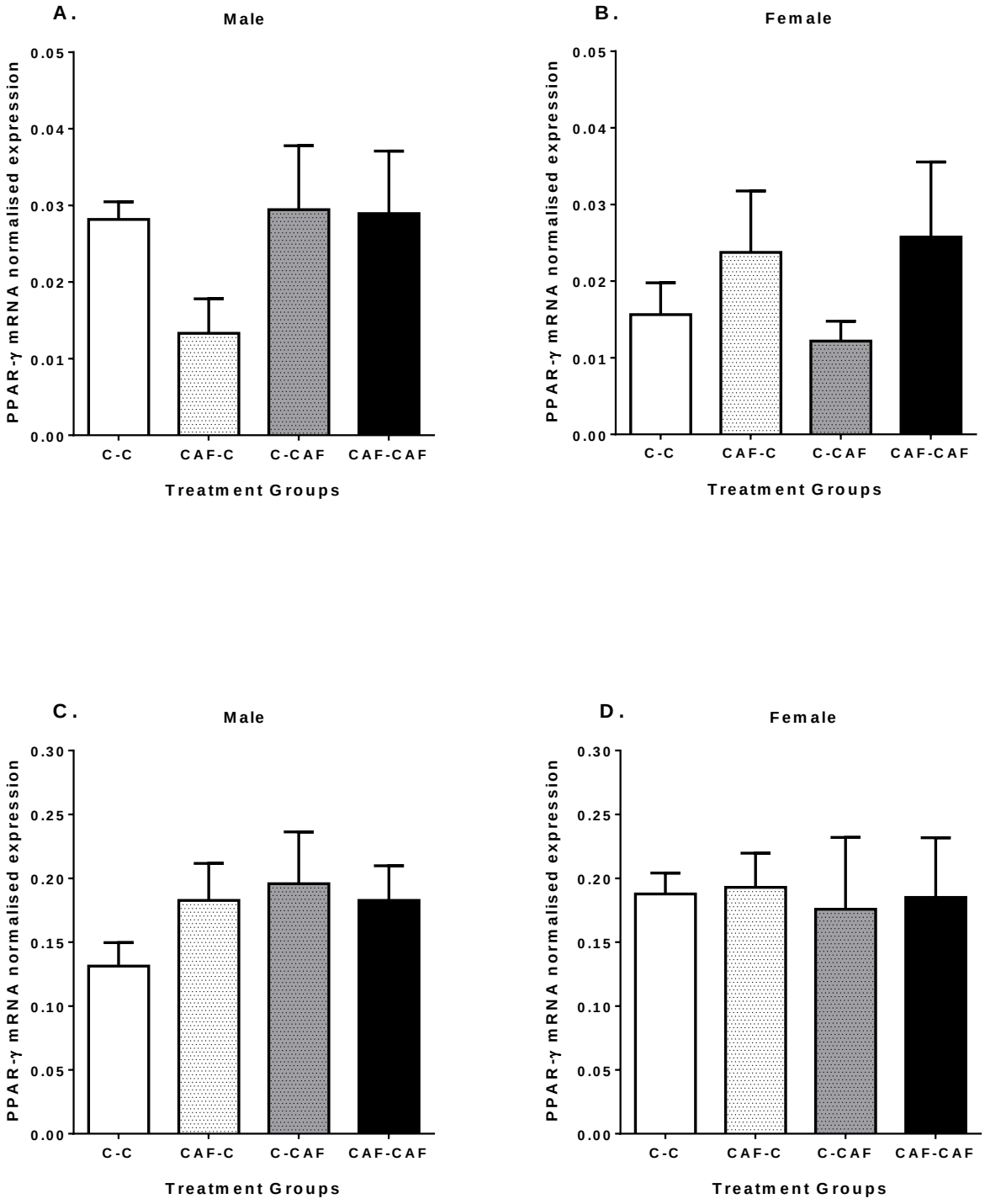


Figure 5.6 The relative expression of PPAR-γ mRNA in subcutaneous (A, B) and retroperitoneal (C, D) adipose tissue in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 months of age. Values are expressed as mean ± SEM.

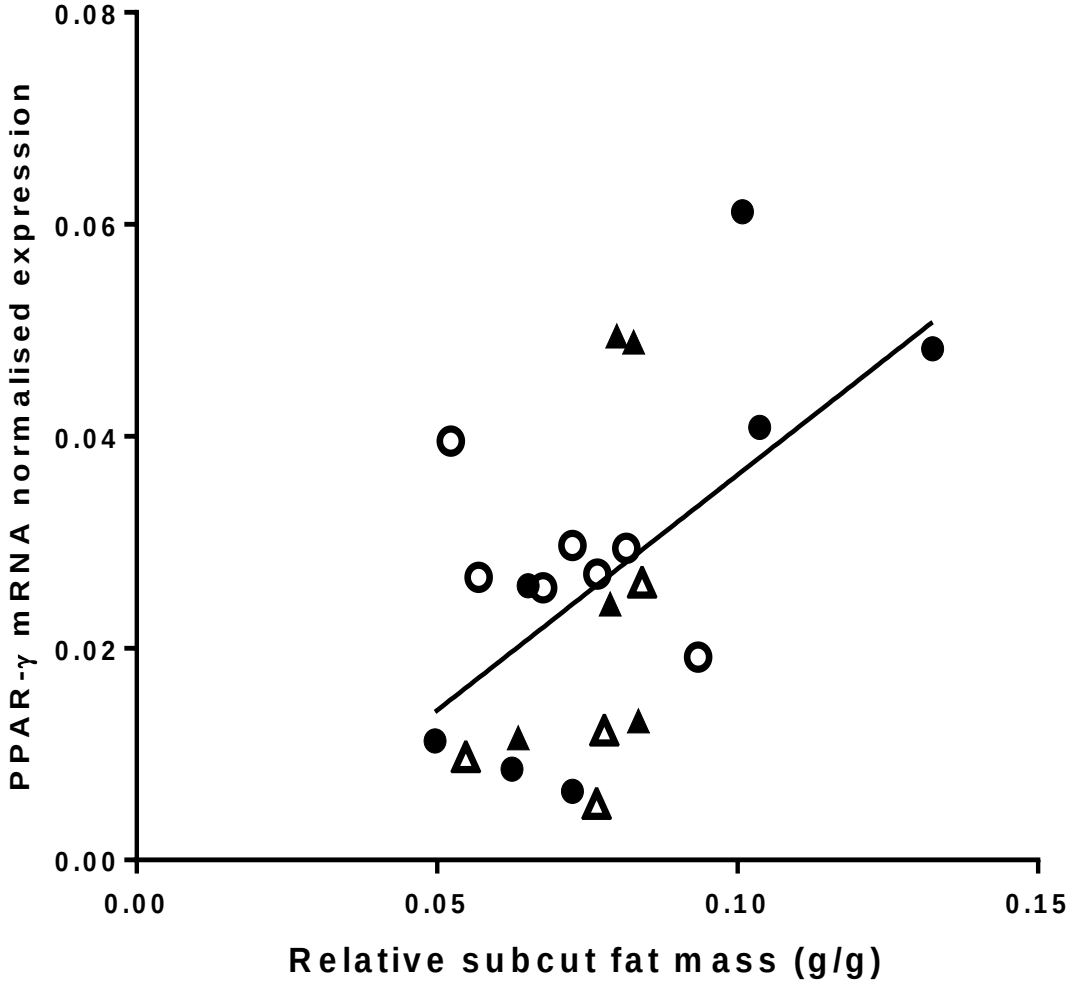


Figure 5.7 The relationship between PPAR-γ mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male offspring at 3 months of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of PPAR-γ mRNA in the subcutaneous adipose tissue and the relative mass of this fat depot in male offspring at 3 months of age. (r^2= 0.279, *P<0.01*).

5.3.5.3 G3PDH and FAS mRNA expression

There was no overall effect of either prenatal or postnatal exposure to a maternal cafeteria diet on G3PDH and FAS mRNA expression in the retroperitoneal depot in either male or female offspring at 3 months of age (Figure 5.8 C, D; Figure 5.10 C, D).

In the subcutaneous fat depot, however, there was a significant interaction between the effect of prenatal and postnatal nutritional exposure in relation to G3PDH mRNA expression in males, such that pups born to CAF dams and suckled by a Control dam exhibited significantly lower G3PDH mRNA expression than those born to and suckled by a Control dam (Figure 5.8 A). FAS mRNA expression in the subcutaneous adipose tissue was significantly higher in male offspring who were born to a CAF dam compared to those born to a Control dam, independent of nutritional exposure during the suckling period (Figure 5.10 A).

There was no effect of either prenatal or postnatal exposure to a cafeteria diet on G3PDH and FAS mRNA expression in the subcutaneous fat depot in female offspring at 3 months of age (Figure 5.8 B; 5.10 B).

There was a significant positive relationship between the expression of G3PDH mRNA in the subcutaneous adipose tissue and relative mass of this fat depot in males at 3 months of age (Figure 5.9). There were no significant correlations, however, between the expression of FAS mRNA in subcutaneous fat and the relative mass of this fat depot in males at 3 months of age. There were also no significant correlations between the expression of G3PDH and FAS mRNA in the subcutaneous adipose tissue and relative mass of this fat depot in females or the expression of G3PDH and FAS mRNA in the retroperitoneal adipose tissue and relative mass of the this fat depot in either males or females at 3 months of age.

There was no relationship between G3PDH or FAS mRNA expression in the subcutaneous adipose tissue and circulating glucose, NEFA or leptin concentrations in either male or female offspring at 3 months of age (Table 5.4). However, there was a significant positive correlation between FAS mRNA expression in the subcutaneous adipose tissue and circulating insulin concentrations in the male offspring at 3 months (Table 5.4). There was no relationship between G3PDH mRNA expression in the subcutaneous adipose tissue and circulating insulin concentrations in either males or females at 3 months of age. There was also no relationship between the expression of either G3PDH or FAS mRNA in retroperitoneal adipose tissue and circulating glucose, insulin or leptin concentrations in either males or females at 3 months of age (Table 5.4). However, both G3PDH and FAS mRNA expression in the retroperitoneal adipose tissue was negatively correlated with plasma NEFA concentrations in males, but not in females, at 3 months of age (Table 5.4).

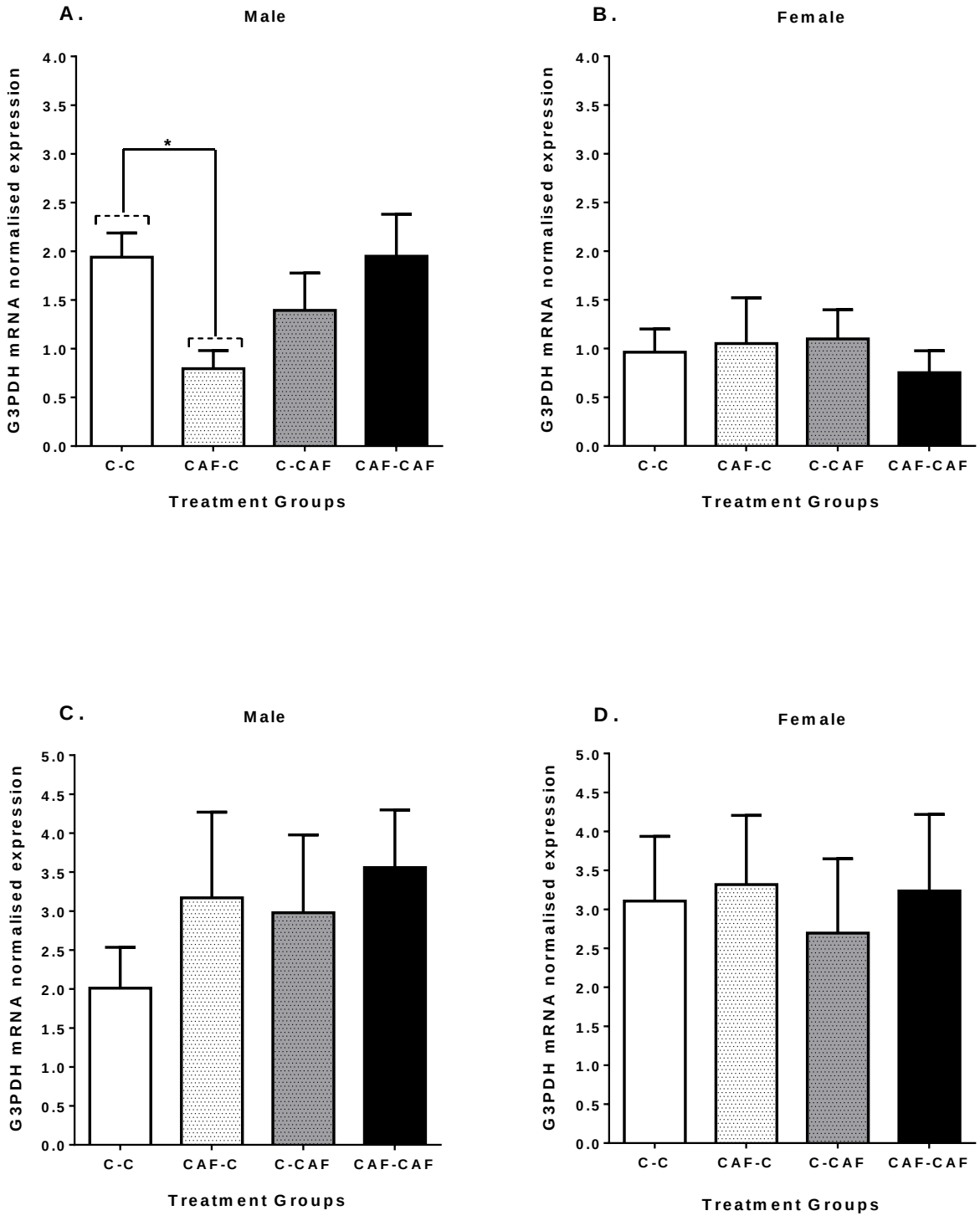


Figure 5.8 The relative expression of G3PDH mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

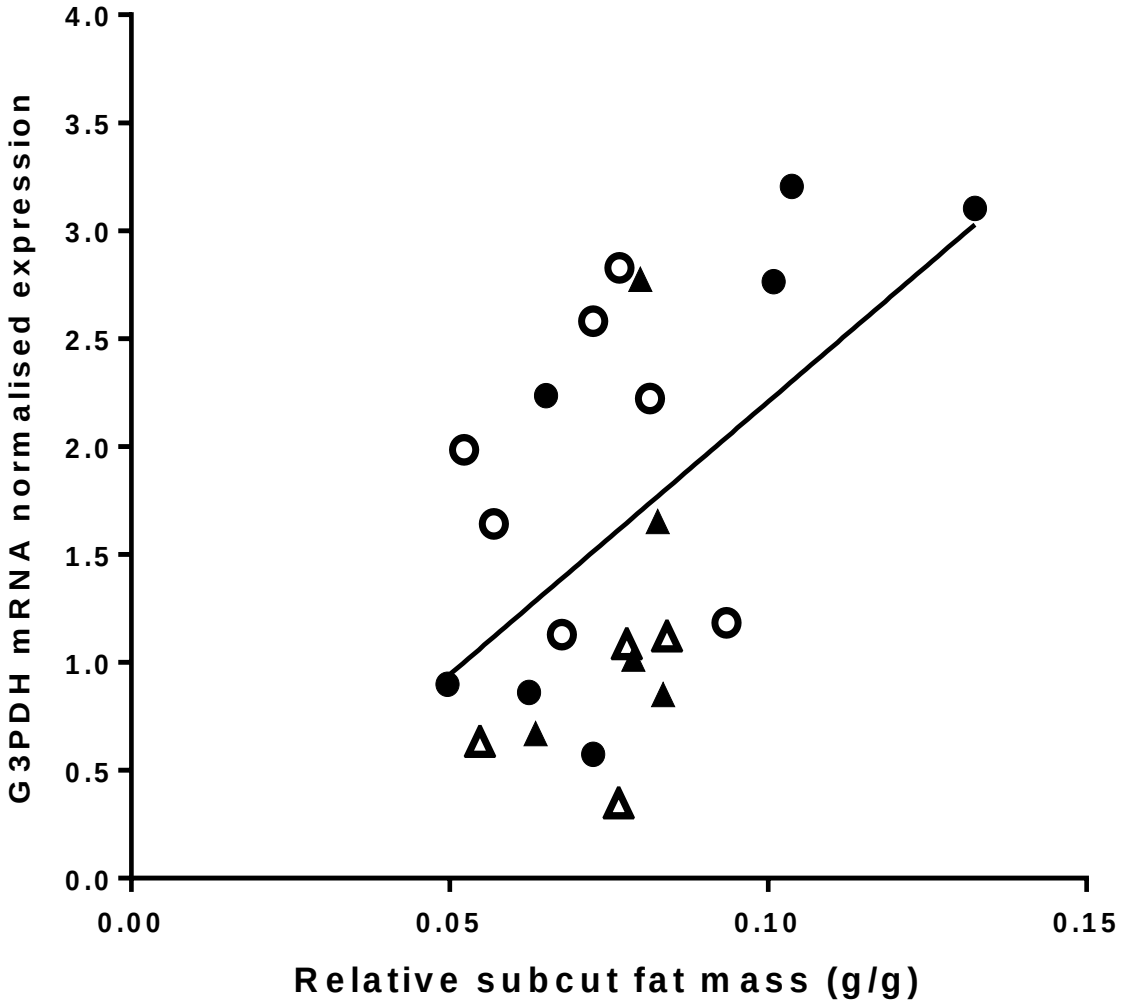


Figure 5.9 The relationship between G3PDH mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male offspring at 3 months of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of G3PDH mRNA in the subcutaneous adipose tissue and the relative mass of this fat depot in male offspring at 3 months of age. (r^2= 0.270, *P<0.05*).

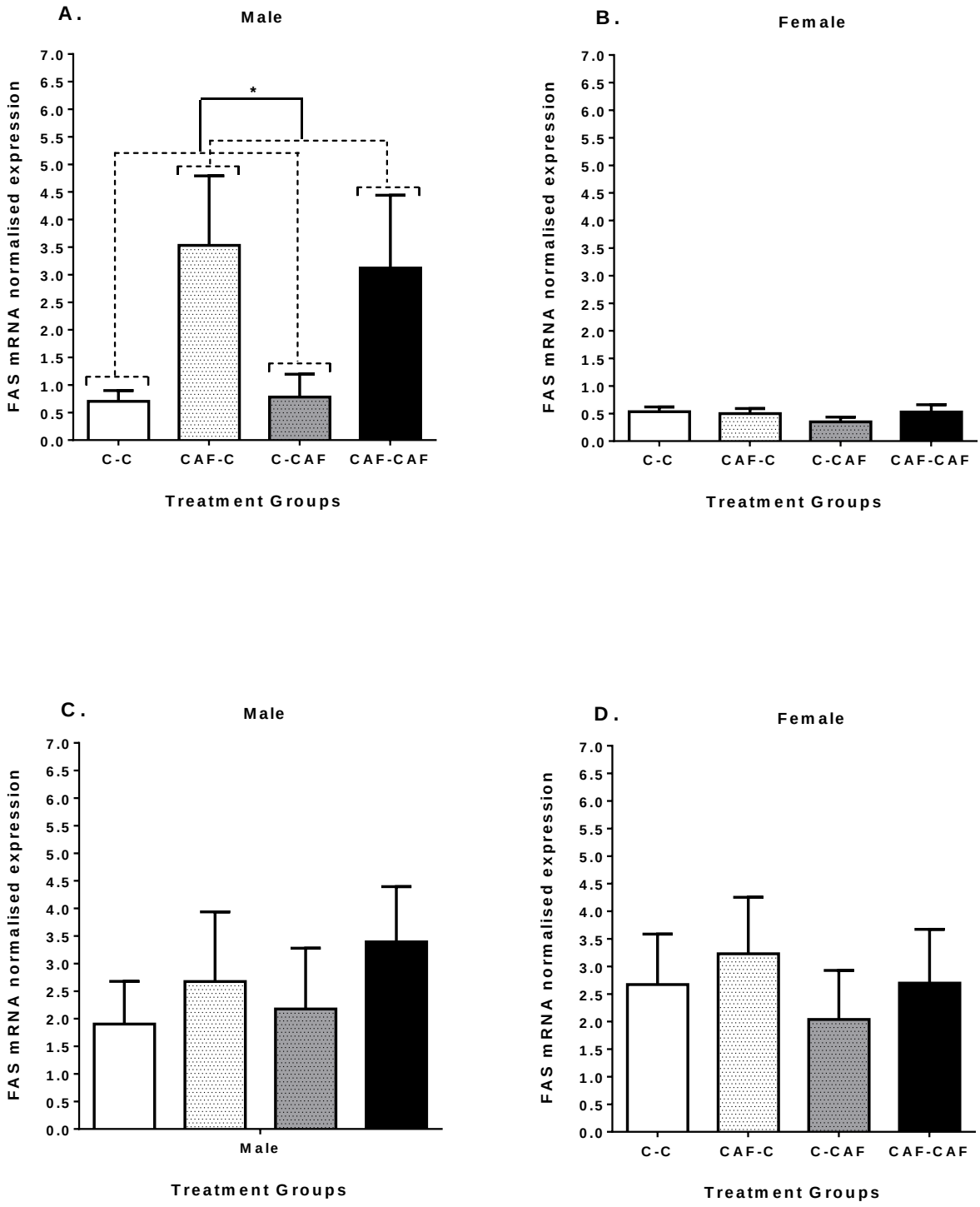


Figure 5.10 The relative expression of FAS mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

5.3.5.4 Adiponectin mRNA expression

There was no effect of either prenatal or postnatal exposure to a cafeteria diet on the relative expression of adiponectin mRNA in the subcutaneous adipose tissue between groups in either males or females (Figure 5.11 A). In the retroperitoneal adipose tissue, however, adiponectin mRNA expression was significantly lower in male offspring who were born to a CAF dam compared to those born to a control dam (Figure 5.11 B) independent of dietary exposure during the suckling period. There were no differences in adiponectin mRNA expression in the retroperitoneal depot between the groups in female offspring (Figure 5.11 B).

There was no relationship between the expression of adiponectin mRNA in the subcutaneous or retroperitoneal adipose tissue and the relative mass of the respective fat depot in either males or females at 3 months of age. The expression of adiponectin mRNA in the retroperitoneal adipose tissue was also not related to plasma concentrations of glucose, NEFA, insulin or leptin in either males or females at 3 months of age (Table 5.4). There was also no relationship between the expression of adiponectin mRNA in the subcutaneous adipose tissue and circulating glucose, insulin or leptin concentrations in either males or females (Table 5.4). There was, however, a significant negative correlation between the expression of adiponectin mRNA in the subcutaneous adipose tissue and circulating NEFA concentrations in the female offspring, but this relationship was not present in males (Table 5.4).

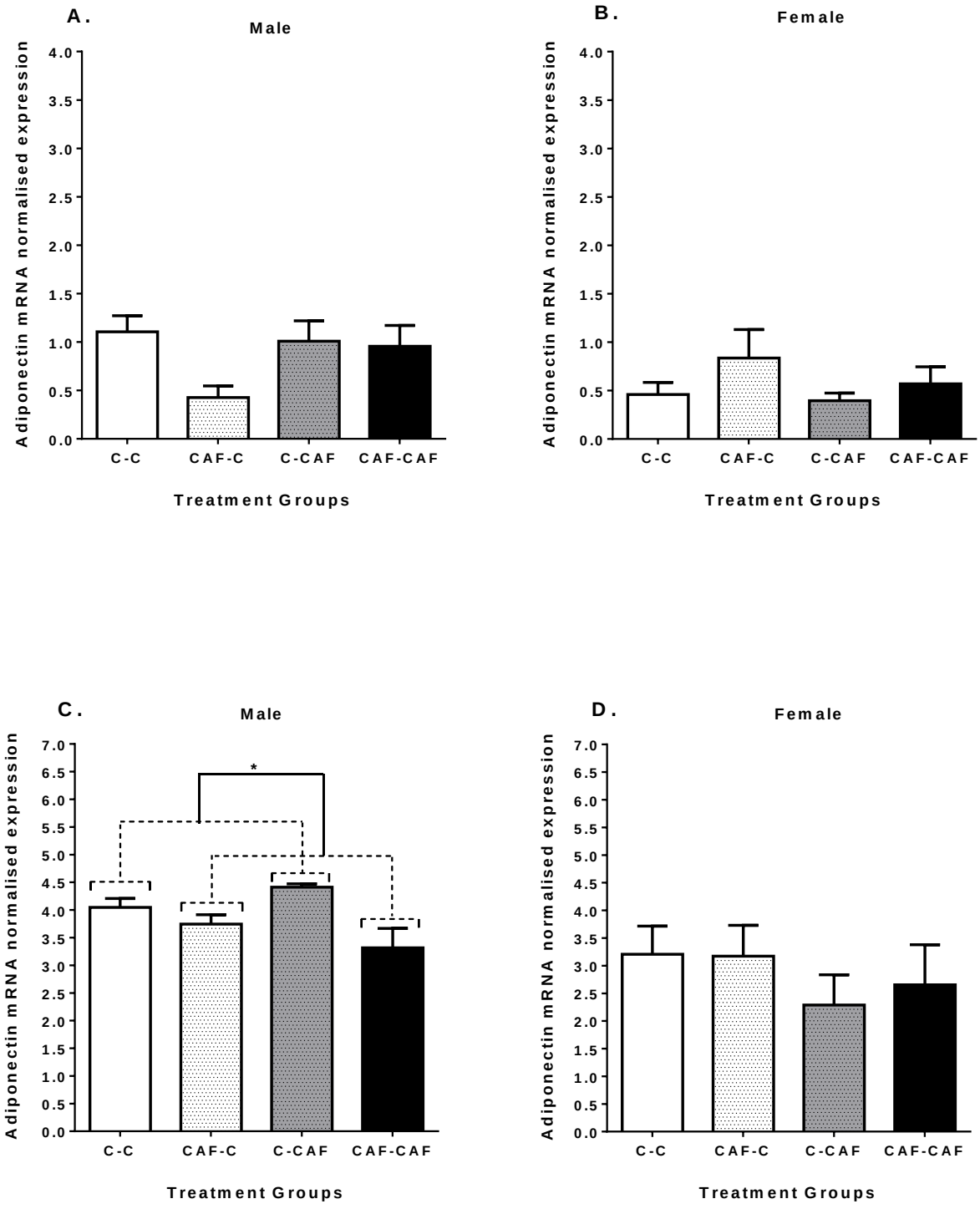


Figure 5.11 The relative expression of adiponectin mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female offspring (B, D) at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05.*

5.3.5.5 Leptin mRNA expression

There was no effect of either prenatal or postnatal exposure to a cafeteria diet on the relative expression of leptin mRNA in the retroperitoneal adipose tissue in either male or in female offspring at 3 months of age (Figure 5.12 A). There was also no difference in the relative expression of leptin mRNA in the subcutaneous adipose tissue between groups in female offspring at 3 months of age. In males, however, there was a significant interaction between the effect of prenatal and postnatal exposure to the CAF diet in relation to leptin mRNA expression in the subcutaneous adipose tissue, such that pups born to CAF dams exhibited significantly lower leptin mRNA expression than those born to controls if they were suckled by a Control dam (Figure 5.12 A).

When the data from all groups were combined there was a significant positive relationship between the expression of leptin mRNA in the subcutaneous adipose tissue and the relative mass of this fat depot in males, but not in females (Figure 5.13). There was no significant relationship between the expression of leptin mRNA in the retroperitoneal adipose tissue and relative mass of this fat depot in either males or females.

The expression of leptin mRNA in the subcutaneous adipose tissue was positively correlated to circulating leptin concentrations in the male offspring, but not in females (Table 5.4), while leptin mRNA expression in the retroperitoneal adipose tissue was positively correlated with circulating leptin concentrations in both sexes (Table 5.4). There was no relationship between the expression of leptin mRNA in either the subcutaneous or retroperitoneal adipose tissue and plasma concentrations of glucose, NEFA or insulin in either males or females (Table 5.4).

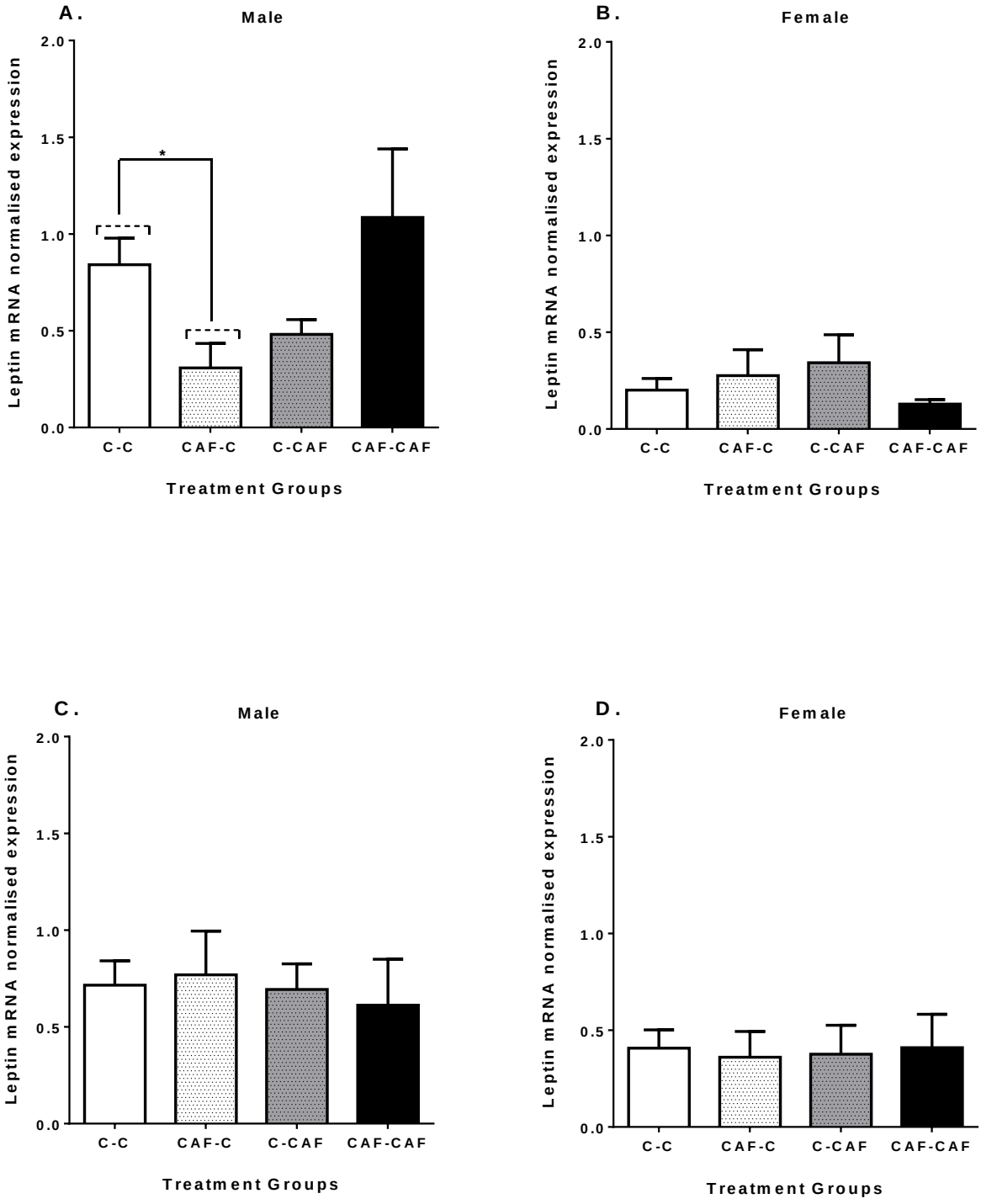


Figure 5.12 The relative expression of leptin mRNA in subcutaneous (A, B) and retroperitoneal adipose tissue (C, D) in the C-C (open bars), CAF-C (open bars with pattern), C-CAF (solid bars with pattern) and CAF-CAF (solid bars) groups of male (A, C) and female (B, D) offspring at 3 months of age. Values are expressed as mean ± SEM. * denotes significance at *P<0.05*.

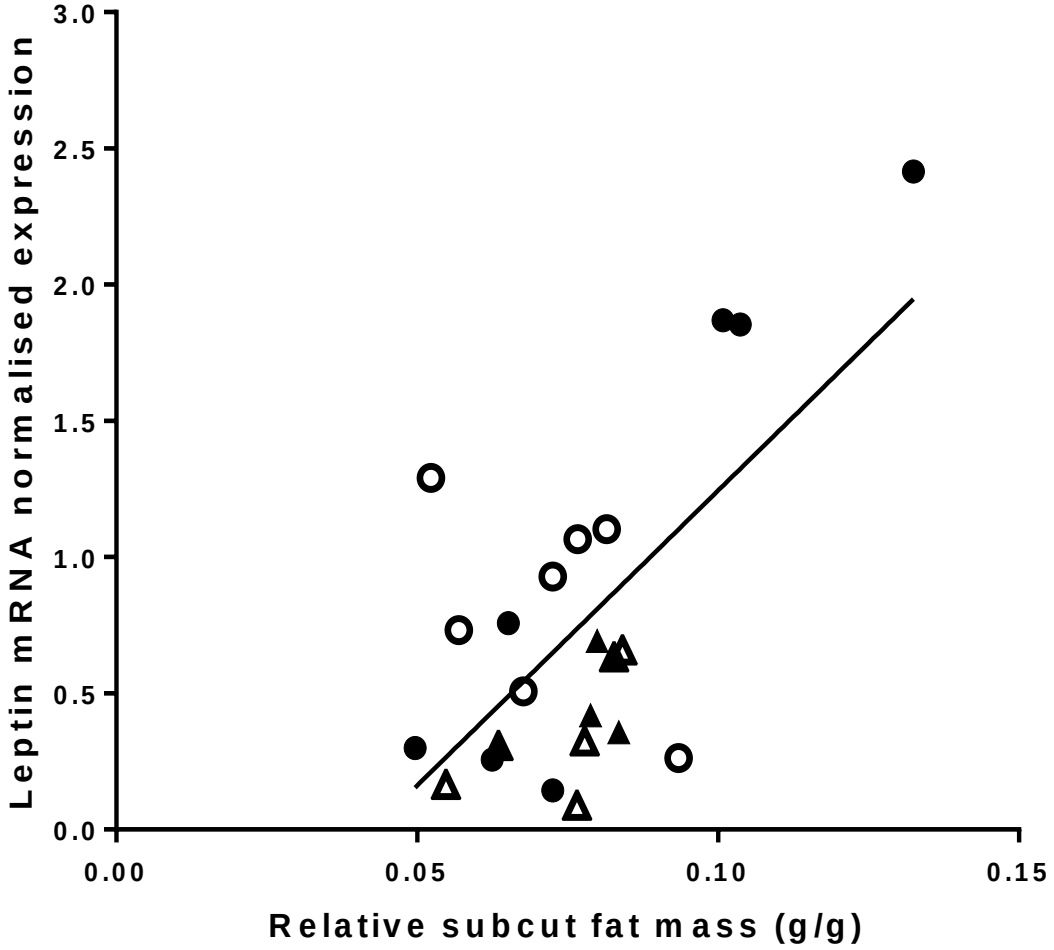


Figure 5.13 The relationship between leptin mRNA expression in subcutaneous adipose tissue and the relative mass of this fat depot in male offspring at 3 months of age. Open circles: C-C group, Open triangles: CAF-C group, Solid triangles: C-CAF group and Solid circles: CAF-CAF group.

There was a significant positive relationship between the expression of leptin mRNA in the subcutaneous adipose tissue and relative mass of this fat depot in male offspring at 3 months of age. (D: r^2= 0.432, *P<0.001*).

5.3.6 Differences in the expression of adipogenic and lipogenic genes between subcutaneous and retroperitoneal adipose tissue in the male and female offspring at 3 months of age

In both males and females, the expression of PPAR-γ, G3PDH and adiponectin were significantly higher in the retroperitoneal depot compared to the subcutaneous depot. In females, but not in males, FAS mRNA expression was also higher, and expression of leptin mRNA tended (*P=0.06*) to be higher in the retroperitoneal compared to the subcutaneous fat depot. The relative expression of SREBP-1c, however, was higher in the subcutaneous adipose tissue compared to retroperitoneal adipose tissue at 3 months of age in female offspring, but this effect was not present in males (Table 5.5).

Table 5.5 The normalised expression of adipogenic and lipogenic genes between subcutaneous and retroperitoneal adipose tissue in the male and female offspring, independent of treatment groups, at 3 months of age. Values are expressed as means ± SEM. ** denotes significance at *P<0.01* and *** denotes significance at *P<0.001*. # denotes a trend at *P=0.06.*

	Male (n = 24)		Female (n = 23)	
	Subcutaneous	**Retroperitoneal**	**Subcutaneous**	**Retroperitoneal**
SREBP-1C	0.01 ± 0.001	0.02 ± 0.002	0.04 ± 0.005	0.016 ± 0.002***
PPAR-γ	0.03 ± 0.004	0.17 ± 0.01***	0.02 ± 0.003	0.19 ± 0.02***
G3PDH	1.73 ± 0.21	2.91 ± 0.40**	0.97 ± 0.15	3.10 ± 0.43***
FAS	1.32 ± 0.38	2.56 ± 0.49	0.48 ± 0.05	2.66 ± 0.45***
Adiponectin	1.00 ± 0.11	3.81 ± 0.15***	0.56 ± 0.09	2.86 ± 0.29***
Leptin	0.82 ± 0.15	0.69 ± 0.09	0.23 ± 0.05	0.39 ± 0.06#

5.4 DISCUSSION

The findings of the present study have demonstrated differing effects of exposure to maternal cafeteria diet during the prenatal and postnatal period on body weight, body fat mass, plasma hormone and metabolite concentrations and expression of key adipogenic and lipogenic genes in the young adult offspring. A key finding was that exposure to the cafeteria diet during the suckling period, independent of dietary exposure before birth, was associated with an increased propensity to diet-induced fat deposition in females. Interestingly, although there appeared to be no significant impact of exposure to a cafeteria diet during the perinatal period on fat deposition after 3 weeks on a high-fat diet in the young adult males, exposure to the cafeteria diet before birth was associated with more significant alterations in the expression of adipogenic and lipogenic genes in males compared to exposure during the suckling period in young adulthood. These findings suggest that early exposure to a high-fat, high-sugar diet has long-term consequences for the regulation of adipocyte gene expression in males, and regulation of adipocyte gene expression and fat deposition in females, and therefore the response of the offspring to a secondary exposure to a cafeteria diet in adult life.

5.4.1 Effect of prenatal and postnatal exposure to the cafeteria diet on offspring growth

Although the male offspring exposed to cafeteria diet either before birth and/or during the suckling period were lighter while consuming the standard chow diet, all offspring caught up in terms of overall body weight during the 3 week period when they had access to the cafeteria diet. These results are consistent with previous studies [93, 117], and suggest that offspring exposed to a high-fat, high-sugar diet at any time during the perinatal period have a higher proportional weight gain when exposed to palatable diets in adulthood. This did not appear to be a consequence of a greater accumulation of fat mass, since fat mass was not different between groups at the end of the 3 week food preference study, which

suggests that lean tissue accumulation was greater in these offspring during this period.

In females, a different pattern was observed, such that there were no differences in body weight between groups during the period they were consuming standard chow, but the offspring of CAF dam who were cross-fostered onto a Control dam at birth were lighter than all other groups after 3 weeks on the food preference diet. This raises the possibility that prenatal exposure to a high-fat diet may be protective against diet-induced weight gain in females, an effect which has also been reported by others [101, 528]. However, it is interesting to note that such growth deficits were not present in those CAF female offspring suckled by another CAF dam and the reasons for this are not clear. The sex specific differences observed in the present study are consistent with previous low protein model studies in which maternal consumption of low protein diet during pregnancy alone has been reported to impact the growth rate to a greater extent in female offspring compared to males and could therefore be related to the lower protein content in the maternal cafeteria diet [442, 529].

5.4.2 The role of prenatal and postnatal nutritional environment as a determinant of the susceptibility to diet-induced obesity in the offspring

The results in this Chapter have shown that exposure to a maternal cafeteria diet during the lactation period was associated with increased fat deposition in young adult female offspring given free access to cafeteria diet for 3 weeks. This suggests that, while the higher fat mass seen in these groups at weaning were normalised by feeding them a standard chow diet for 3 weeks post-weaning (as shown in Chapter 2), offspring exposed to the cafeteria diet during the suckling period retained a greater susceptibility towards fat deposition during a secondary exposure to the high-fat, high-sugar diet in postnatal life. This effect was independent of the diet their mother had consumed during pregnancy, demonstrating the dominant role of maternal cafeteria diet during the suckling period in the programming of susceptibility to diet-induced obesity in the female

offspring. In contrast to the observations at 3 weeks, the observed increase in the fat mass in the female offspring at 3 months appeared to be related more closely to increases in visceral than subcutaneous adiposity. This is significant, since increased visceral fat accumulation is well-established to be more detrimental to metabolic health outcomes [530-532]. It is also important to note that this fat accumulation occurred in the absence of a higher food intake in these female offspring during this period [533], suggesting that the effect is due to differences in the responsiveness of fat cells to increased nutrition and/or a reduction in overall metabolic rate, rather than differences in energy intake. This increased susceptibility to diet-induced obesity was not observed in those CAF female offspring suckled by a Control dam, suggesting that improved nutrition in the early postnatal period has the potential to prevent the long lasting detrimental effects of exposure to high-fat, high-sugar cafeteria diet before birth. While exposure to a high-fat diet during lactation has been associated with an increased adiposity in the adult offspring compared to those exposed to control diet during lactation [106, 534], the present study is one of the few studies which demonstrated the importance of maternal diet during lactation in programming an increased susceptibility to diet-induced obesity in the offspring.

In contrast to females, exposure to the cafeteria diet during the perinatal period did not appear to influence the susceptibility to diet-induced fat deposition in the male offspring. While not all previous studies have produced consistent results, there is evidence that sexual dimorphism exists in the susceptibility to diet-induced obesity in response to maternal overnutrition/obesity, such that females are typically more susceptible than males [102, 117, 393]. Therefore, the results of this study add to the current body of evidence suggesting that the impact of exposure to a cafeteria diet during the perinatal period on subsequent obesity risk is greater in female offspring.

5.4.3 The effect of prenatal and postnatal maternal diet on plasma hormones and metabolite concentrations at 3 months of age

In the present study, plasma leptin concentrations were higher in young adult female offspring suckled by CAF dams compared to those suckled by controls, consistent with the higher relative fat mass in this group. Plasma leptin concentrations were also directly correlated with relative total fat mass and other major fat depots in both males and females, which is consistent with the established role of leptin as a peripheral signal of body fat stores in both humans and animals [438, 439]. Previous studies have reported that prenatal nutrition can shape future susceptibility to diet-induced obesity by altering postnatal leptin sensitivity, and these studies identified peripheral leptin resistance as a key mechanism that can influence postnatal susceptibility to diet-induced obesity in female offspring exposed to prenatal undernutrition [535]. Whilst leptin resistance was not directly assessed in the present study, the fact that the relationship between leptin concentrations and fat mass appeared to be consistent across all treatment groups suggests that there were no substantial differences in leptin sensitivity between groups. The higher food intake in the male offspring of CAF dams during the food preference period [533] may be indicative of a reduce sensitivity to the appetite-suppressing effects of leptin in these animals, although this will need to be confirmed directly.

The results from the present study also demonstrated that perinatal exposure to a cafeteria diet impacted on plasma insulin concentrations in the adult offspring in a sex-specific manner. Although plasma insulin levels did not correlate with relative total fat mass and other major fat depots in both males and females, female offspring born to CAF dams had higher plasma insulin concentrations, in the absence of higher plasma glucose, compared to those born to Control dams, independent of dietary exposure during suckling period. The occurrence of elevated insulin concentrations at any given level of glucose provides evidence of reduced insulin sensitivity, however, this needs to be confirmed by direct assessment of insulin sensitivity in future studies. These results therefore imply that the effect of cafeteria diet exposure *in utero* on glucose-insulin metabolism

is sex-specific, with females being more susceptible than males. Previous studies have reported sex-specific alterations in the insulin signalling pathway in the skeletal muscle of adult offspring of dams fed cafeteria diets during pregnancy and lactation with female offspring of cafeteria-fed dams exhibiting impaired Akt (also known as 'protein kinase B') phosphorylation, suggesting an impaired phosphoinositide 3-kinase (PI3K) activity and providing molecular evidence of impaired insulin signalling [536]. It therefore appears that exposure to a cafeteria diet before birth may have adverse effects on glucose-insulin metabolism following a secondary exposure to a cafeteria diet in adult life in females, but not in males, and that this effect cannot be reversed by nutritional interventions applied in the suckling period.

In addition to the effects on insulin, female offspring born to CAF dams also had lower plasma NEFA concentrations at 3 months of age. This may be a consequence of the higher insulin concentrations, since it has been reported previously that infusion of insulin lowers plasma NEFA concentrations by suppressing NEFA release from adipose cells [537]. An alternate possibility, however, is that the lower plasma NEFA concentrations were due to increased uptake of fatty acids into adipose tissue, however this seems unlikely given that it was those female offspring suckled by CAF dams, rather than those exposed to the cafeteria diet before birth, who had the highest relative fat mass at the end of the 3 week period of access to the cafeteria diet in young adulthood.

5.4.4 Effect of prenatal and postnatal nutrition on the expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal tissue at 3 months of age

5.4.4.1 SREBP-1c mRNA expression

SREBP-1c mRNA expression in the subcutaneous adipose tissue was significantly elevated in male offspring of CAF dams after 3 weeks of access to the cafeteria diet compared to offspring of controls. It is notable that this

increased expression of SREBP-1c mRNA was observed in the absence of any differences in subcutaneous fat mass between the groups. The increased expression of SREBP-1c mRNA reported in the present study was different to the effects observed in male offspring at 3 weeks (Chapter 4), at which time SREBP-1c mRNA was lower in those offspring exposed to the cafeteria diet during the suckling period and negatively related to body fat mass. While we did not observe any relationship between SREBP-1c mRNA and fat mass in the present study, we did identify a positive relationship between SREBP-1c mRNA expression in the subcutaneous fat depot and plasma insulin concentrations at 3 months of age in the male offspring. This is consistent with previous studies suggesting that SREBP-1c mRNA expression is positively regulated by insulin [474, 477, 478]. However, given that there were no differences in plasma insulin concentrations between the treatment groups in males, this cannot account for the higher SREBP-1c mRNA expression in offspring of CAF dams observed in this study. The lack of a significant relationship between SREBP-1c mRNA expression and relative subcutaneous fat mass is consistent with previous studies which have demonstrated that expression of SREBP-1c is not necessarily linked to lipogenesis, and that targeted disruption of the SREBP-1 gene has a limited effect on lipogenic gene expression in adipose tissue [480]. The reason why the effects on SREBP-1c mRNA expression were only seen in male offspring is unclear. Although one previous study reported a higher SREBP-1c mRNA expression in the liver of female rats compared to males [538], no previous studies have attempted to study the effect of sex on SREBP-1c mRNA expression in adipose tissue. Therefore, the mechanisms underlying SREBP-1c mRNA mediated nutritional programming and the reason(s) for sex-specific differences in the impact of perinatal exposure to a cafeteria diet on the expression of this gene in later life requires further research.

5.4.4.2 PPAR-γ mRNA expression

Unlike SREBP-1c, PPAR-γ mRNA expression after 3 weeks of consuming a cafeteria diet was similar between groups in both subcutaneous and retroperitoneal fat depots in male or female offspring. This observation was in

contrast to the observations in Chapter 4 and suggests that the expression and regulation of the PPAR-γ gene was not programmed by perinatal exposure to a high-fat and high-sugar cafeteria diet, even following secondary exposure to the cafeteria diet in adulthood. The positive correlation between PPAR-γ and subcutaneous fat mass observed in the male offspring at 3 months of age does, however, support the role of this adipogenic and lipogenic factor in promoting lipid storage in mature adipose cells, consistent with previous studies [483-486]. Interestingly, I did not observe a significant correlation between PPAR-γ expression and subcutaneous fat mass in female offspring in the present study, suggesting that the role of PPAR-γ in regulating lipid storage in adult fat depots is different between males and females. While the central role of PPAR-γ in adipocyte differentiation has been firmly established in previous studies [483-486], the lack of significant relationship between PPAR-γ mRNA expression and relative fat masses in the female offspring at 3 months suggests that the increased total relative fat mass in these offspring is likely to have been programmed via a PPAR-γ independent mechanism. Although differences between the sexes in the impact of maternal high-fat/junk-food feeding on adipose tissue deposition and on adipocyte gene expression have been reported in previous studies [117, 400, 494, 495], these studies have generally found more pronounced effects in females compared to males, consistent with the findings in Chapter 4. The results from the present Chapter, however, found more pronounced effects in males compared to females. Thus, further studies will be needed to investigate the sex specific regulatory mechanisms for the PPAR-γ gene and its role in the susceptibility to diet-induced obesity following perinatal overnutrition.

5.4.4.3 G3PDH and FAS mRNA expression

The present study revealed a significant interaction between the prenatal and postnatal nutritional environment in relation to the expression of G3PDH mRNA in the subcutaneous fat depot in males, such that, for offspring suckled by Control dams, those born to a Control dam had higher expression of G3PDH than those born to CAF dams. In the case of FAS, offspring born to CAF dams

had significantly elevated FAS mRNA expression in the subcutaneous fat depot at 3 months of age, independent of dietary exposure during the suckling period. The observations from the present study therefore suggest that the expression of G3PDH and FAS mRNA in young adulthood is dependent, at least in part, on dietary exposure during the perinatal period.

As discussed in Chapter 4, it has been demonstrated previously that dietary intake can influence G3PDH mRNA expression [500]. Thus, one possible explanation for the reduced G3PDH mRNA expression in those CAF male offspring suckled by a Control dam compared to those both born to and suckled by Control dam is that the programmed changes in the expression of G3PDH mRNA as a consequence of exposure to CAF diet before birth, cannot be reversed by a nutritionally balanced diet during the suckling period, and an secondary exposure to an increased supply of fat and/or sugar in young adulthood would acted to reduce the mRNA expression of this gene in these CAF animals. Previous studies have also demonstrated that G3PDH mRNA expression is reduced in the adipocytes of obese compared to lean individuals [501], suggesting that the expression of this gene is negatively regulated by increased lipid accumulation in fat depots. Whilst the mechanisms underlying the sex-specific differences in the effect of perinatal exposure to the cafeteria diet on expression levels of G3PDH mRNA are unclear, the direct relationship between G3PDH mRNA expression in the subcutaneous fat depot and relative mass of this fat depot in males is consistent with the established role of this gene in promoting lipid storage [275, 499]. Interestingly, the difference in G3PDH mRNA expression between the groups were not associated with any differences in fat mass between the groups, however whether this might emerge when the offspring are maintained on the cafeteria diet for more extended periods is unknown.

The increased expression of FAS mRNA in the male offspring of CAF dams, along with its direct relationship with plasma insulin concentrations, suggests that insulin may be a mediator of the increased FAS mRNA expression [265, 304, 305]. However, as plasma insulin concentrations were not different

between the treatment groups in males, this cannot entirely explain the increased FAS expression in offspring of CAF dams observed in this study. Since SREBP-1c positively regulates FAS mRNA expression [477], it is possible that the increased FAS mRNA expression in offspring of CAF dams was a consequence of the increased SREBP-1c mRNA expression in these offspring. One possible explanation, at least in part, for the increased expression of FAS gene in CAF male offspring is that the programmed changes to the expression of these genes, SREBP-1C and FAS in particular, as a consequence of exposure to a CAF diet before birth, cannot be reversed by being exposed to a nutritionally balanced diet in the suckling period. Interestingly, the differences between offspring groups in FAS mRNA expression were distinct from those at 3 and 6 weeks of age; at 3 weeks of age, there was no effect of exposure to the cafeteria diet either before birth or during the suckling period on FAS mRNA expression in either subcutaneous or retroperitoneal adipose tissue and at 6 weeks of age, FAS mRNA expression was increased in the retroperitoneal adipose tissue of male offspring born to CAF dams who were suckled by a Control dam. As discussed in the previous Chapter, FAS mRNA expression in adipose tissue is highly sensitive to nutritional status [291, 297, 298] and is markedly upregulated by consumption of a fat-free diet [299, 300, 303] and down-regulated by a high-fat diet [275, 498]. It is therefore possible that the expression of FAS mRNA increased to a greater extent in the offspring of CAF dams compared to offspring of controls during the time they were on the low-fat standard chow, and that this effect was not completely reversed by secondary exposure to the cafeteria diet in adulthood. However, since we did not collect tissue samples immediately before the period of cafeteria diet feeding, we cannot confirm this possibility in the present study.

5.4.4.4 Adiponectin mRNA expression

Adiponectin mRNA concentrations were lower in the retroperitoneal adipose tissue of male offspring born to CAF dam at 3 months of age, independent of their dietary exposure during the suckling period. Interestingly, this pattern of expression was similar to that seen in female offspring at the 6 week time point.

Previous studies have reported that maternal high-fat diets during gestation and lactation play a key role in adiponectin mRNA expression in adipose tissue and plasma adiponectin levels, and that this is associated with an increased risk of insulin resistance and other metabolic disorders in the adult offspring [539, 540]. Although plasma adiponectin levels are reported to be lower in adult obese individuals compared to lean individuals [353, 354, 362], no differences in fat mass were observed in male offspring at the time of tissue collection in the present Chapter. Therefore the reason for this lower adiponectin mRNA expression is unclear. As discussed in Chapter 4, one possible explanation, at least in part, for the reduced expression of adiponectin mRNA in the CAF male offspring in the retroperitoneal fat depot is that the regulation of adiponectin mRNA expression in this fat depot could be permanently programmed by exposure of pre-adipocytes to the high-fat/high-sugar diet *in utero.* It is therefore possible that these programmed alterations cannot be reversed by cross-fostering them onto a Control dam and result in a greater reduction in adiponectin gene expression during exposure to a high-fat/high-sugar diet in adult life. Since reductions in adiponectin expression and secretion are known to be associated with reduced insulin sensitivity [358, 511, 541, 542], it is tempting to speculate that the lower adiponectin expression in male offspring of CAF dams would be associated with greater diet-induced decreases in insulin sensitivity in these offspring. However, since insulin sensitivity at 3 months of age was not directly assessed in the present study further studies are required to test this hypothesis. It is also unclear why the effects on adiponectin mRNA expression were not observed in females. While previous studies have reported sex differences in the expression of adiponectin, with females having a higher levels of adiponectin expression in adulthood, the regulation of adiponectin expression and secretion is reported to be complex and dependent on factors other than gonadal steroids [508-510], further studies would be needed to investigate these underlying mechanisms.

5.4.4.5 Leptin mRNA expression

It has been well-established in previous studies that circulating leptin concentrations are increased in obese human subjects [439, 543-545] and

positively correlated with relative body fat mass in both humans and animals [513, 546, 547]. Thus, the direct relationships between leptin mRNA expression with body fat mass and plasma leptin concentrations in males and the positive correlation of leptin mRNA expression in the retroperitoneal fat mass with plasma leptin concentrations in both male and female offspring in the present study are consistent with previous studies. These data also suggest that the increased leptin mRNA expression and circulating leptin concentrations in female offspring suckled by CAF dams were a consequence of the increased fat accumulation in the adipose depots of these animals. This same explanation cannot, however, be applied to explain the reduced leptin mRNA expression in the subcutaneous adipose tissue of male CAF offspring suckled by a Control dam compared to Control offspring suckled by a Control dam, since there were no differences in overall or subcutaneous adiposity in these animals. It is notable that this pattern of leptin mRNA expression was similar to that observed for G3PDH mRNA expression in the male offspring in this same group of animals. Given that G3PDH plays an important role in lipid synthesis, this may suggest that prenatal exposure to a cafeteria diet can program both lipid and leptin synthetic capacity in this fat depot. As with G3PDH mRNA, it is unclear why this effect was not observed for offspring of CAF dams who were also exposed to the cafeteria diet during the suckling period; however the results suggest that a mismatch between the nutritional environments experienced before birth and in early postnatal life may be important in mediating these effects.

5.4.5 Differential expression of adipogenic and lipogenic genes in subcutaneous and retroperitoneal adipose tissue in male and female offspring at 3 months of age

Consistent with observations at 3 weeks and 6 weeks of age reported in Chapter 4, SREBP-1c mRNA was still more highly expressed in the subcutaneous depot in females, but not in males, at 3 months of age. This pattern of SREBP-1c mRNA expression, i.e. lower levels in visceral adipose tissue compared to subcutaneous adipose tissue, is consistent with previous reports in both male and female human adults [472]. The increased expression of PPAR-γ, G3PDH,

FAS, adiponectin and leptin in retroperitoneal compared to subcutaneous adipose tissue in females at 3 months of age was consistent with the pattern seen in the female offspring at 6 weeks of age (Chapter 4). Males exhibited a different pattern in the expression of key adipogenic and lipogenic genes at 3 months compared to 6 weeks, such that the expression of PPAR-γ, G3PDH and adiponectin was higher in the retroperitoneal adipose tissue compared to subcutaneous adipose tissue at 3 months. Previous studies have reported that the rate of adipocyte hypertrophy is lower in subcutaneous compared to retroperitoneal adipose tissue in young adult rodents [526], which is consistent with the lower expression of key adipogenic and lipogenic genes in the subcutaneous adipose depot at 3 months of age in this study.

It is important to note that there was a ~4-5 fold increase in the weight of fat mass in the retroperitoneal fat depot from weaning to 3 months of age, whereas the increase in subcutaneous fat depot was only ~1-2 fold, independent of treatment group, in both males and females (data not shown). This suggests a greater degree of adipocyte hypertrophy in the retroperitoneal fat depot compared to subcutaneous fat depot during this period; which would be consistent with the higher expression of key adipogenic and lipogenic genes in this fat depot in the offspring at 3 months of age. As discussed in Chapter 4, subcutaneous and retroperitoneal adipose depots have different roles in the regulation of whole body energy balance. The results from the present study therefore suggest that there would have been higher rates of lipogenesis and adipocyte hypertrophy in the retroperitoneal fat compared to subcutaneous fat in response to a 3 week high-fat/high-sugar cafeteria diet in both male and female offspring and this may account, at least in part, for the increased expression of key adipogenic and lipogenic genes in the retroperitoneal adipose tissue in these offspring at 3 months of age. As mentioned earlier, in contrast to the observations at 3 weeks, the observed increase in fat mass in the female offspring at 3 months appeared to be related more closely to increases in visceral than subcutaneous adiposity. It is therefore possible that the female adipocytes have increased lipogenic capacity and lipid synthesis [525] and the increased expression of key adipogenic and lipogenic genes in the

retroperitoneal fat depot in the female offspring could possibly account for the deposition of more visceral fat mass in these offspring. However, the mechanisms leading to this differential sensitivity of visceral and subcutaneous fat depots are less clear and further investigation would be needed to better understand the mechanisms involved in such changes.

5.5 SUMMARY

In summary, the results of this Chapter have demonstrated that exposure to increased maternal nutrition during the suckling period programs an increased susceptibility to diet-induced obesity in the female offspring upon re-exposure to cafeteria diet post-weaning. Importantly, those female offspring exposed to the cafeteria diet *in utero* who were suckled by a control dam were resistant to this effect, suggesting that a nutritionally balanced diet during suckling period can prevent the adverse effects of exposure to a high-fat, high-sugar cafeteria diet before birth on susceptibility to diet-induced obesity in female offspring in later life. This study also reported that the increased fat mass in young adulthood was not restricted to increase in subcutaneous fat depot but also to increases in fat mass of visceral depots and, as majority of adverse metabolic effects of obesity are the result of excess visceral adipose tissue [530-532, 548], this is likely to have negative consequences for the metabolic health of the offspring. Interestingly, however, there appeared to be limited impact of the maternal cafeteria diet on adipocyte gene expression in females, and the reason for this is unclear.

The reverse was observed in the male offspring, with more significant effects on adipocyte gene expression at the end of the 3-week period of exposure to the cafeteria diet in adulthood in the absence of an increased susceptibility to fat deposition. This Chapter also reported sex specific differences in the relative contribution of prenatal and postnatal nutritional environment on these effects, with exposure to the cafeteria diet during the suckling period producing greater adverse effects in females, while exposure to the cafeteria diet before birth appeared to be more important in males. This may suggest differences in the

critical windows of development for the key systems regulating fat deposition/energy balance in males and females, and implies that the timing of nutritional interventions aimed at re-programming the offspring may be sex specific. It is however, clear that further studies are required to increase our understanding of what factors lead to the differences between sexes and fat depots, including epigenetic effects, to help us to understand the link between overnutrition during critical periods of development and disease risk in adulthood.

CHAPTER 6

CHAPTER 6: GENERAL DISCUSSION

The increasing number of women entering pregnancy overweight or obese has led to a growing need to understand the impact of maternal obesity/nutritional excess on the long-term health of the offspring [93, 117, 394-396]. Numerous studies had modelled maternal obesity/nutritional excess by means of a number of different approaches and a wide range of epidemiological, clinical and experimental animal studies had demonstrated that maternal obesity, maternal hyperglycemia or maternal intake of diets high in fat, sugar or total calories during pregnancy and lactation was associated with an increased risk of obesity and metabolic disease in the offspring in postnatal life [15-17, 399, 400]. However, the majority of studies conducted prior to this thesis had examined the impact of exposure to maternal obesity/maternal high-fat diets during both the prenatal and early postnatal periods [15-17]. Although a handful of studies had provided clues that the impact of exposure to this obesogenic environment during these two periods of development may not be equivalent, the results were not consistent. For example, one study reported that exposure to a high-fat diet during the early postnatal period was more important for metabolic programming than exposure before birth [106], while another study reported prenatal exposure to high-fat feeding was both necessary and sufficient to program growth and fat mass in the offspring [116]. Therefore, data on the separate contributions of exposure to nutritional excess during the prenatal and early postnatal periods were lacking. Therefore, the central aim of this thesis was to determine, using a cross-fostering approach in a rat model, the relative contribution of exposure to a maternal cafeteria diet during the prenatal and suckling periods to the metabolic outcomes of the offspring, specifically body weight, fat mass and, the expression of key adipogenic and lipogenic at weaning, in early adolescence and in young adulthood.

The aim of the first experimental Chapter (Chapter 2) of this thesis was to determine the relative contribution of the prenatal and early postnatal nutritional

environment to fat deposition and glucose tolerance in the offspring at weaning and at 6 weeks of age. The primary aim of this Chapter was to identify the critical window of development during which exposure to the cafeteria diet would have the greatest influence on offspring body weight, adiposity and insulin sensitivity, using a cross-fostering approach. The results of this study showed that the exposure to high-fat/high-sugar cafeteria diet during the suckling period resulted in increased fat mass and elevated leptin levels in both male and female offspring at weaning independent of whether they were born to a dam consuming a control or high-fat/high-sugar diet. The results of this Chapter also provided evidence that the adverse effects of exposure to a high-fat/high-sugar diet before birth could potentially be reversed by nutritional interventions applied during the suckling period. This study also showed that when all offspring were weaned onto a control low-fat chow diet, the offspring suckled by CAF dams, which had been shown to be fatter at weaning, were no longer fatter than those suckled by Control dams at 6 weeks of age, and there were also no differences in glucose tolerance between the groups at this age. This therefore suggested that the impact of the early nutritional environment on fat mass in the offspring can be overcome, or at least controlled, by consuming a nutritionally balanced diet after weaning.

The results of Chapter 2 suggested that nutritional exposures during the suckling period were a more important determinant of growth and fat deposition at weaning compared to the nutritional environment before birth. Since milk is the predominant source of nutrition for the offspring during this time, I hypothesised that changes in the milk composition, in particular the level and type of fats, would have an important role in mediating the effects of the maternal cafeteria diet on offspring fat deposition. This was supported by previous studies which had demonstrated that, of all the components of the maternal diet, the amount of fat and the content of individual fatty acid classes are most closely reflected in their levels in breast-milk [190, 191, 402, 405-407]. However, while previous studies had reported increases in the total fat content of milk from rat dams fed a high-fat diet during pregnancy and lactation [147, 148], no studies had determined the impact of maternal cafeteria diets on milk

fatty acid composition or how this related to metabolic outcomes in the offspring. The aim of Chapter 3, therefore, was to determine the impact of a cafeteria diet during the lactation period on the fat and protein content and fatty acid composition of the milk, fatty acid status of their offspring and to determine whether any changes were related to the fat mass of the offspring at weaning. The results from this study showed that the higher maternal intakes of total fat, saturated fat, trans fats and n-6 PUFA were reflected by higher levels of these components in the milk, and that levels increased in direct proportion to maternal intake. These results were in line with previous reports of a dose-dependent relationship between total fat content [147], trans fatty acid levels [203, 450, 451] and n-6 PUFA content [139, 140] in the maternal diet and levels in the milk, and suggested that the same is true of saturated fat. The results from this study also showed that the fatty acid composition of the maternal milk translated into persistent effects on the fatty acid status of the offspring, particularly for the n-3 LCPUFA and trans fatty acids. I identified strong positive relationships between the levels of specific fatty acids, in particular saturated, trans fat and n-6 PUFA, in the maternal milk and relative fat mass in the offspring at weaning, which supported the hypothesis that changes in the fatty acid composition as a result of maternal cafeteria diet intake plays an important role in mediating the effects of maternal diet on offspring fat mass, and may well account for the higher adiposity at weaning in offspring suckled by CAF dams. The results from Chapter 3 add to the growing body of evidence that not only the total fat intake, but the levels of specific fatty acids in the diet, play a critical role in offspring fat deposition [141]. This is the first study to demonstrate the impact of providing dams with a cafeteria diet on the fatty acid composition of the milk and the fatty acid status of the offspring at and after weaning, and to identify specific fatty acids that appear to play a particularly important role in driving fat deposition during the suckling period.

Taken together, the outcomes of the first two experimental Chapters of this thesis suggested that the suckling period is a critical window of development during which exposure to maternal cafeteria diet can program increased adiposity in the offspring, however the underlying mechanisms remained

unclear. In Chapter 4 of this thesis I therefore aimed to determine the effect of exposure to a cafeteria diet during the prenatal and/or suckling periods on adipocyte gene expression in the offspring. Previous studies had shown that the exposure to increased maternal nutrition or increased glucose concentrations during critical windows of development resulted in increased adipocyte proliferation and differentiation and increased expression of key adipogenic and lipogenic genes in adipose cells [91, 117, 235, 257, 375, 400]. However, these studies did not consider the relative importance of exposure to an increased nutrient supply before birth and during early postnatal period for the programming of altered expression of these adipocyte regulatory genes. In Chapter 4, I hypothesised that the increased fat accumulation in the offspring suckled by CAF dams at weaning was a consequence of increased expression of adipogenic and lipogenic genes in the adipose tissue of the offspring, which persisted after birth and drove increased fat deposition in visceral and subcutaneous fat depots. Thus, the aim of Chapter 4 of this thesis was to determine whether the increased fat mass in offspring exposed to a cafeteria diet during the suckling period was due to altered expression of genes regulating adipogenesis and lipogenesis in the subcutaneous and/or retroperitoneal fat pads. Chapter 4 also aimed to determine whether any changes in adipogenic, lipogenic and adipokine gene expression in the adipose tissue of these offspring persisted after the pups have been fed on a standard chow diet for 3 weeks after weaning.

The results demonstrated that exposure to increased maternal nutrition during the suckling period resulted in altered expression of key adipogenic and lipogenic genes, particularly in the subcutaneous adipose depot. This study also demonstrated sex differences in these effects with more pronounced effects in females. I showed that the exposure to the cafeteria diet during the suckling period, independent of dietary exposure before birth, resulted in increased mRNA expression of the lipogenic gene, PPAR-γ, in subcutaneous fat in female offspring at weaning. This finding was consistent with previous studies in sheep [375] and rodents [490], and suggested that an upregulation of PPAR-γ mRNA

in key adipose depots may be a mechanism through which maternal cafeteria diet exposure increases adiposity at weaning, at least in female offspring.

An unexpected finding from this study was that the mRNA expression of the lipogenic transcription factor SREBP-1c at weaning was down-regulated in both male and female offspring who were suckled by dams fed the cafeteria diet, independent of dietary exposure before birth, and was still down-regulated in the subcutaneous depot of female offspring at 6 weeks of age. While these findings were contrary to our expectations, given that SREBP-1c has been implicated in promoting fat storage, this finding was consistent with previous studies in humans [471-474] which demonstrated a decreased level of SREBP-1c mRNA in obese subjects. I also found a significant negative correlation between SREBP1-C mRNA expression and fat mass in both subcutaneous and retroperitoneal fat at weaning, thus providing further evidence that the reduction in the expression of SREBP-1c mRNA in obese individuals/animals is likely to reflect a compensatory response to limit further fat deposition, as speculated by authors of previous rodent studies [312, 475].

Another important finding of this Chapter was that adiponectin mRNA expression at weaning was significantly increased in at least one fat depot in both male and female offspring who were exposed to the maternal cafeteria diet during the suckling period. This observation was different to what would be expected based on findings in adults that adiponectin concentrations are negatively correlated with BMI and fat mass [353, 354, 362]. However, previous studies from humans and animals had suggested that the relationship between adiponectin concentrations and fat mass is different in infants and young children compared with older children and adults [233, 369, 370, 502]. Studies in humans reported a higher plasma adiponectin level with positive correlations with neonatal body weight/fat mass in neonates compared to adults [369, 370] and studies in sheep reported an increased expression of adiponectin mRNA in the adipose tissue of fetal sheep with direct relationship to the mean fractional growth rate of lambs across the first 30 days of postnatal life [233]. As previous rodent studies had reported adiponectin increases lipid accumulation in

adipocytes by suppressing lipolysis and increasing adipocyte differentiation [505, 506], one possibility is that the increased expression of adiponectin observed in this Chapter, may in fact be contributing to the increased fat deposition in offspring suckled by cafeteria-fed dams

Interestingly, the majority of the gene expression changes observed in the offspring suckled by cafeteria-fed dams did not persist after the offspring had been fed on a nutritionally balanced chow for 3 weeks after weaning. This suggested that these changes were unlikely to contribute to long-term programming of fat deposition, at least as long as rats were maintained on a nutritionally balanced chow diet. This was the first study to demonstrate the relative importance of exposure to maternal cafeteria diet before birth and during the early postnatal period on the expression of adipocyte regulatory genes in the adipose tissue of the offspring during early development. The results suggested that an increase in nutrient supply during the early postnatal period was more important than the prenatal period for fat deposition, and that increased fat deposition observed in the offspring exposed to maternal cafeteria diet during suckling period may be (at least in part) due to increased adipogenic and lipogenic expression in adipose tissue, particularly in females.

While relative fat mass was no longer different between groups at 6 weeks of age, after all offspring had been consuming a standard nutritionally balanced chow diet since weaning, I identified several differences between groups in adipocyte expression at 6 weeks of age, suggesting that the potential of these cells to store lipid may be persistently altered by exposure to the cafeteria diet during the perinatal period. It was unknown, however, whether the differences in adipocyte gene expression at 6 weeks had functional consequences for the lipogeneic capacity of the adipose depots in these offspring in adulthood and whether these changes would result in an altered susceptibility to fat deposition and weight gain if access to a cafeteria diet was re-introduced during the juvenile period. In Chapter 5 of this thesis, I investigated the hypothesis that offspring exposed to a maternal cafeteria during the suckling period would exhibit an exaggerated response to exposure to a cafeteria diet in postnatal life,

both in terms of the fat deposition and expression of key adipogenic, lipogenic and adipokine genes within the adipose tissue, independent of their nutritional exposure before birth. In Chapter 5, I showed that exposure to a maternal cafeteria diet during the suckling period was associated with an increased susceptibility to diet-induced fat deposition in the female offspring upon re-exposure to cafeteria diet post-weaning. Importantly, this study showed that those female offspring exposed to the cafeteria diet *in utero* who were suckled by a control dam were resistant to this effect, suggesting that a nutritionally balanced diet during the suckling period can potentially prevent the adverse effects of exposure to a high-fat, high-sugar cafeteria diet before birth on susceptibility to diet-induced obesity in the female offspring in later life. While previous studies reported that exposure to a high-fat diet during lactation is associated with increased adiposity in the adult offspring compared to those exposed to control diet during lactation [106, 534], Chapter 5 of this thesis is the first study to directly compare the effects of prenatal and postnatal exposure to the cafeteria diet on susceptibility to diet-induced obesity in the offspring.

Interestingly, although there appeared to be no significant impact of exposure to a cafeteria diet during the perinatal period on fat deposition after 3 weeks on a high-fat diet in males, exposure to the cafeteria diet before birth was associated with altered expression of adipogenic and lipogenic genes in males in young adulthood. The findings from this study therefore suggested that the critical windows of development for the key systems regulating fat deposition/energy balance may be different between males and females, indicating that the timing of nutritional interventions aimed to re-program the offspring may be sex specific. The results of this Chapter also showed that the increased fat mass in female offspring suckled by CAF dams in young adulthood was present in visceral, as well as subcutaneous, fat depots. Since the majority of the adverse metabolic effects of obesity are the result of excess visceral adipose tissue [530-532, 548], this is likely to have negative consequences for the metabolic health of the offspring later in life and longer term follow up of offspring will be an important area for future study.

An important aspect of this thesis was the fact that the responses of both males and females were examined in all experiments, and sex differences in the programming of obesity in response to the maternal cafeteria diet during perinatal period have been consistently demonstrated throughout the thesis. In Chapter 2, I saw a sex-dependent regulation of plasma insulin and leptin concentrations and fat deposition in response to the maternal cafeteria diet during the perinatal period at weaning and at 6 weeks of age. Results from Chapter 3 showed that female, but not male, offspring exposed to the cafeteria diet during the suckling period had lower levels of both n-3 and n-6 PUFA in RBC phospholipids at weaning, while male, not female offspring, suckled by CAF dams had lower levels of n-3 LCPUFA at 6 weeks of age. This points to the presence of differences in the metabolism of n-6 and n-3 PUFA during the neonatal period between males and females, and that the impact of altered maternal fat intake on fatty acid in the offspring will be different for males and females. This Chapter also showed a positive relationship between LA and total n-6 PUFA in maternal milk and relative fat mass in male, but not female offspring, at weaning which implies that the effect of n-6 PUFA on adipogenesis/lipogenesis in the fetal/early postnatal period may be sex-specific.

In Chapter 4, I demonstrated that female offspring suckled by CAF dams exhibited an increase in PPAR-γ gene expression in the subcutaneous adipose depot at weaning and female offspring born to a CAF dam exhibited a lower expression of adiponectin mRNA in the retroperitoneal adipose depot at 6 weeks of age, effects that were not observed in males. Chapter 4 also showed sex-specific differences in the pattern of expression of key adipogenic and lipogenic genes between fat depots, independent of treatment group, at weaning and at 6 weeks of age. In Chapter 5, I observed that exposure to cafeteria diet during the suckling period programmed an increased susceptibility to diet-induced obesity in young adulthood only in females. This study also demonstrated that perinatal exposure to a cafeteria diet impacted on plasma insulin and NEFA concentrations in the adult offspring in a sex-specific manner. In Chapter 5, I also showed altered expression of adipogenic and lipogenic genes in males born to CAF dams in young adulthood, which were not observed in females

suggesting sex-specific differences in the critical windows of development for the key systems regulating fat deposition/energy balance between the sexes. This emphasises the need to exercise caution when extrapolating results from experiments conducted only in male offspring to both males and females, and the importance of including both male and female offspring in studies of metabolic programming.

Taken together the results presented in this thesis suggest that while exposure to a cafeteria diet during the suckling period has a greater impact on the expression of key adipogenic and lipogenic genes and adiposity in the early postnatal period and increased susceptibility to obesity in females than it does in males, exposure to cafeteria diet before birth appeared to be more important in the expression of key adipogenic and lipogenic genes in males in young adulthood. Very few studies to date have compared the effects of palatable diets on adiposity and on expression of key adipogenic and lipogenic genes between males and females. Despite the fact that females have been reported previously to be more susceptible to diet-induced obesity in response to maternal overnutrition/obesity [102, 117, 393], our current understanding of the mechanisms underlying the sex-specific programming of obesity and expression of key genes involved in adipogenesis and lipogenesis remains limited. It has been speculated that interactions between cafeteria diets and sex hormones may be playing a role in the regulation of key adipogenic and lipogenic genes, since previous studies have suggested that both estrogen and androgens have a role in regulating the expression of key adipogenic and lipogenic genes in adipocytes [386, 491-493]. It is also possible that differences in lipid metabolism in the fat depots of males and females [494, 495] may contribute to sex differences in the expression of key adipogenic and lipogenic genes in response to exposure to the cafeteria diet during critical windows of development.

Although previous studies had reported sex differences in the expression of key adipogenic and lipogenic genes, it appears that the regulation of expression and secretion of these genes is complex and that it is likely to be dependent on other factors in addition to gonadal steroids [508-510, 549]. The potential epigenetic

effects on the genomes, such as the methylation/acetylation modifications are also referred in relation to DOHaD phenomena [549]. Therefore, the mechanisms underlying the sex specific regulatory mechanisms for the key adipogenic and lipogenic genes in the programming of obesity in response to exposure to increased maternal nutrition during the prenatal/suckling period represents an important area for further research.

Although the studies in this thesis have utilised a rodent model for the study of metabolic programming, considering the implications of findings in this thesis for clinical practice, it should be noted that rats and mice deposit very little fat before birth and that the suckling period has been identified as a critical time window for fat deposition in rodents. Unlike rats, however, human infants deposit significant fat stores before birth, which potentially indicate that late gestation period is particularly important in fat deposition in human infants and lactation period in rodents is approximately equivalent to late gestation period in human infants. Therefore, it is important to exercise caution when attempting to extrapolate the results from rodent studies to humans. The results presented in this thesis do, however, raise the possibility that improving the quality of the maternal diet in late pregnancy could potentially reduce the risk of excess fat deposition in human infants who are exposed to an excess nutrient supply earlier in gestation.

There are a number of additional studies that could build on the results of this thesis and assist in interpreting some of the key findings. First, it will be important in future studies to assess glucose tolerance and insulin sensitivity in the offspring at multiple time points after birth, and both before and after the re-introduction of the high-fat/high-sugar cafeteria diet. This is particularly important given that previous studies [358, 511, 541, 542] have indicated that expression of some of the key adipogenic and lipogenic genes is associated with insulin sensitivity. Another approach to strengthen the current data would be to conduct an additional set of post-mortems immediately before providing the offspring with the cafeteria diet in the postnatal period, which would enable us to assess whether the changes in adipocyte gene expression between groups at the end

of the cafeteria-diet feeding period were a cause or consequence of exposure to the high-fat diet. It would also be beneficial to conduct metabolic tests, including measures of fat mass and glucose tolerance/insulin sensitivity, in the offspring after a longer period of exposure to the high-fat diet to determine and compare the metabolic response to long-term exposure to the cafeteria diet in the different experimental groups.

The results from this thesis clearly indicate that mothers consuming poor-quality diets, high in trans, saturated fats and n-6 PUFA, and lower in n-3 LCPUFA, are likely to be exposing their offspring lower n-3 LCPUFA levels and elevated levels of n-6 PUFA, saturated and trans fats during critical windows of development. Given that elevated exposure to saturated fats and n-6 PUFA in early life has been implicated in increasing the later risk of obesity and other metabolic and allergic diseases, and given the well-described negative impacts of elevated trans fat intake on cardiovascular and metabolic health in adults [462, 463], an important area for future research will be to identify the potential negative long-term health impacts of exposure to an increased supply of n-6 PUFA and trans fatty acids in early life including their role in metabolic programming and programming of obesity in early life. This thesis also suggests that developmental ontogeny of the key systems regulating fat deposition/energy balance is likely to be sex-specific and implies that the timing of nutritional interventions aimed to re-programme the offspring may be different in males and females. Looking specifically at what factors lead to the sex-specific differences in fat deposition, including epigenetic effects during critical windows of development, to gain a better understanding of the mechanisms which underlie the sex-specific effects observed will be an important expansion of the studies presented in this thesis.

The findings from this thesis add to the growing body of evidence that exposure to the maternal cafeteria diet during the early postnatal period is a more important determinant for adiposity at weaning than exposure before birth. Importantly, the data from this thesis suggest that a nutritionally balanced diet during the early postnatal period can prevent the negative effects of exposure to

a high-fat/high-sugar cafeteria diet in the prenatal period. Furthermore, this thesis has demonstrated that the effects of being exposed to a high-fat/high sugar cafeteria diet during the perinatal period on offspring adiposity can be reversed/controlled by providing access to a nutritionally balanced diet post-weaning. The findings from this thesis further suggest that mothers consuming poor-quality diets, high in trans, n-6 PUFA and saturated fats, and lower in n-3 LCPUFA, are likely to be exposing their offspring lower n-3 LCPUFA levels and elevated levels of n-6 PUFA, saturated and trans fats during critical windows of development and the fatty acid composition of the milk supply during the suckling period may be an important determinant of fat deposition during this period. The findings of this thesis also demonstrated that exposure to a cafeteria diet during the early postnatal period alters the expression of key adipogenic and lipogenic genes in the adipose tissues of the offspring and that this period represents a critical window for the programming of obesity.

This thesis has also provided evidence that the relative contribution of the nutritional environment during prenatal and postnatal periods in the programming of susceptibility to diet-induced obesity were sex-specific, suggesting that the timing of nutritional interventions aimed to re-program the offspring may be different in males and females. The studies from this thesis have presented novel and important insights into the mechanisms underlying the early origins of obesity and provided a basis for future research to further understand the possible mechanisms driving this programming. This current research is an important step towards developing nutritional guidelines for pregnant and lactating women who are overweight and obese to ensure optimal long-term outcomes for their infants.

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