THE EFFECTS OF CAFETERIA DIET FEEDING ON MATERNAL ADAPTATION TO PREGNANCY, GROWTH AND DEVELOPMENT OF FETUS AND GLUCOSE HOMEOSTASIS LATER IN LIFE

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Declaration

I declare that the contents of this thesis are my own work. All studies in this thesis performed under the supervision of Professor Simon Langley-Evans and Dr Sarah McMullen, Department of Nutiritional Sciences, Nottingham University, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD.

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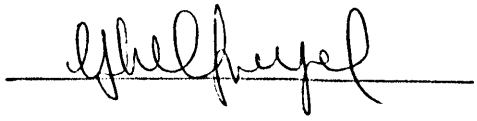

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Dedication

This thesis, I dedicate to my mum and dad

Publications arising from this thesis

Peer-Reviewed Papers

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Conference Abstracts

Nutrition Society – Nottingham UK, July 2008. The effect of cafeteria diet feeding on maternal body composition and plasma volume expansion during early gestation (poster presentation)

Society for Endocrinology – Harrogate UK, March 2009. The effect of cafeteria diet on maternal adaptation to pregnancy during pre, early and late gestation (poster presentation)

Fetal and Neonatal Physiological Society – Winchester UK, July 2010. Maternal and postnatal diet impact upon glucose homeostasis in rats (oral presentation)

Abstract

Both epidemiological and experimental studies have demonstrated adverse effects of maternal obesity upon both maternal and fetal health. It has been shown that feeding a cafeteria diet in rat pregnancy can induce altered food preferences and greater weight gain in the resulting offspring. This study firstly aimed to examine the effect of diet-induced obesity, or high fat feeding on maternal adaptation to pregnancy and fetal growth. Forty-eight 3-week-old virgin female Wistar rats were randomly divided into two groups fed control (n=24) and cafeteria diet (n=23). 8 weeks later, all rats were mated and after confirming pregnancy half of the rats fed cafeteria diet switched to control, and half of the control rats switched to the cafeteria diet. Maternal cafeteria diet feeding prior to pregnancy resulted in profound adiposity, but did not compromise physiological adaptation to pregnancy or impact upon reproductive success. Maternal cafeteria diet feeding during pregnancy only or maternal obesity induced distinct effects on fetal growth, with pre-gestational obesity resulting in reduced fetal weight at 20 days gestation. In contrast, cafeteria diet feeding during pregnancy only resulted in increased fetal weight. The main focus of the study was the development of the fetus, and consideration of whether maternal obesity, or cafeteria diet feeding during pregnancy determined body weight, body composition, metabolic biomarkers and glucose homeostasis throughout life. With respect to this point, the same study design was used with additional nutritional challenges during lactation and post-weaning. In contrast to the literature, offspring exposed to cafeteria diet at any stage pre-weaning, showed no evidence of

hyperphagia or increased adiposity. Adult offspring exposed to cafeteria diet in early life and weaned onto chow diet, had low fasting glucose and insulin concentrations and were more sensitive to insulin during an i.p. glucose tolerance test. When weaned onto cafeteria diet, offspring exhibited glucose intolerance. There was evidence that rats arrived at a glucose intolerant state via different mechanistic routes which were dependent on the feeding regime during lactation. The data suggested that components of the insulin signalling pathway may be targets for programming by maternal obesity, but that IRS2 and Akt2 do not play major roles. There was also evidence of pancreatic insufficiency in some groups of animals that were fed cafeteria diet during lactation. The observations in this study confirm that maternal over-nutrition and obesity during pregnancy are risk factors for metabolic disturbance in the resulting offspring. The effects on glucose homeostasis were independent of offspring adiposity. However the programming of a glucose intolerant phenotype was dependent upon consumption of cafeteria diet during the post-weaning period.

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Abbreviations

11 ß-HSD-2	11 ß-hydroxysteroid dehydrogenase type 2
ANOVA	Analysis of variance
BMI	Body Mass Index
COV	Coefficient of variaton
CHD	Coronary heart disease
FAO	Food and Agriculture Organization
FABP	Fatty acid binding protein
Foxo	Forkhead box protein
FTO	Fat mass and obesity associated gene
GDM	Gestational Diabetes Mellitus
GLUT4	Glucose transporter type 4
GSK3	Glykogen synthase kinase-3
IGF1	Insulin like growth factor-1
IGF2	Insulin like growth factor-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IR	Insulin receptor
IRS1	Insulin receptor substrate type 1
IRS2	Insulin receptor substrate type 2
LGA	Large-for gestational age
MC4R	Melanocortin receptor 4
MUFA	Monounsaturated fatty acids

NCD	Non-communicable diseases
PAR	Predictive Adaptive Response
PDK	Phosphoinositide-dependent kinase
PEPCK	Phosphoenol pyruvate carboxykinase
PI3K	Phosphotidylinositol 3-kinase
PKB/AKT	Protein kinase B
PKC	Protein kinase C
PPAR γ2	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
RAS	Renin-angiotensin system
SPSS	Statistical package for social sciences
TNF- α	Tumor necrosis factor- α
UCP1	Uncoupling protein 1
UCP3	Uncoupling protein 3
WHO	World Health Organization

1.0 INTRODUCTION

1.1 Obesity

1.1.1 Definition of obesity

The World Health Organization (WHO) defines obesity as a major public health problem in accordance with its current global epidemic prevalence and the serious physical, psychological and social effects associated with overweight *(World Health Organization, 2000).* It is reported that more than 300 million adults around the world are obese and more than 1 billion are overweight *(Krishnamoorthy et al., 2006).* According to the estimates of the WHO there will be about 2.3 billion overweight people aged 15 years and above, and over 700 million obese people worldwide by 2015 *(WHO Fact Sheet, 2000).*

Obesity is defined as abnormal or excessive fat accumulation that may impair health *(WHO Fact Sheet, 2006).* In 1869, Quetelet observed that amongst a large group of individuals weight varied roughly in proportion to the square of the height *(Fryn, 2003).* Since then this ratio has been known as Body Mass Index (BMI) which is universally accepted to diagnose adulthood overweight and obesity at the population level. If the BMI is greater than the normal, then the person is overweight, and conversely the person is underweight if the BMI is low. Table 1.1 shows the classification of obesity.

Table 1. 1 Classification of obesity

Classification	BMI (kg/m^2)	Risk of co-morbidities
Underweight	<18.5	Low
Normal	18.5-24.9	Average
Overweight	25.0-29.9	Increased
Obese:	>30.0	
Class 1	30.0-34.9	Moderate
Class 2	35.0-39.9	Severe
Class 3	>40.0	Very severe

(WHO Technical Report Series, 2000)

The increase in body mass in obesity largely represents an accumulation of fat. However, the use of BMI does not distinguish the mass of fat and muscle tissues *(Garrow, 1981).* It has been suggested that measuring intra-abdominal or central fat accumulation may be a more powerful method to define the risk of health complications associated with obesity *(Klein et al., 2007).* In addition to this, current cut off points of BMI were shown to mislead the determination of overweight in certain ethnic groups *(Dudeja et al., 2001).* It is generally accepted that these cut off points should be lower for Asians since the metabolic risks are greater than Caucasians *(Pan and Yeh, 2008).*

The cut off points of obesity in adults are not the same in children. To define overweight in children reference population based measurements are used such as gender specific age-growth charts *(de Onis, 2004).* For children and teenagers ages to 2 to 19 years, overweight is defined as the 95th percentiles and risk of overweight is defined between the 85th and 95th percentiles *(Ogden, 2004).*

1.1.2 Aetiology of Obesity

The aetiology of obesity is multi-factorial *(Fryn, 2003).* It involves complex interactions between different environmental and social factors, hormones and genetic background.

The WHO defined the fundamental cause of obesity as an energy imbalance between calories consumed on one hand and calories expended on the other *(WHO Technical Report Series, 2000).* The most important attributable factors within this point of view which contribute to current trend in prevalence are listed as:

1- Global shift in diet towards increased intake of energy dense foods, that are high in fat and sugars but low in vitamins, minerals and other micro nutrients.
2- A trend towards decreased physical activity due to the increasingly sedentary nature of many forms of work, changing models of transportation and increasing urbanization.

Over the past three centuries human's environmental factors and nutritional habits have been shifting *(Popkin, 2001).* The recent increasing trend of obesity and related complications has been identified as the manifestation of this transition. During the Paleolithic era patterns of human diet and physical activity levels were optimal. It has been shown that compared with modern Western diets, the Palaeolithic diet contained consistently higher protein and polyunsaturated fatty acids (*Kuipers et al.,*

2010). Beyond the Palaeolithic era, when the modern agricultural period emerged and gave rise to the energy-dense Western pattern diet the health status of humans worsened *(Popkin, 2006, Gardner and Rhodes, 2009).* Firstly, mostly in developed countries and increasingly in developing countries, consumption of processed, high-fat foods, sugary desserts and refined grains dramatically increased *(Heidemann et al., 2008, Gilbert and Khokhar, 2008).* In addition to this, foods that are high in fiber, vitamins and minerals were replaced by processed versions. These changes in diet lead to emergence of non-communicable diseases (NCD) such as hypertension, diabetes, cardiovascular disease, cancer and the metabolic syndrome. Increased portion sizes and snacking on these kinds of foods have been associated with positive energy balance and obesity *(de Graaf, 2006, Mela, 2006).* Secondly, the displacement of very physically active hunter-gatherer lifestyle into sedentary, office based lifestyle contributed to the development of chronic diseases *(O'keefe et al., 2010).* It was concluded that a physical activity level of 1.75 (490 kcal/day) approximates to that of Paleolithic times *(Eaton and Eaton, 2003).*

While the evidence for the contribution of environmental and life style factors in the development of obesity is abundant and strong, the evidence for a role of genetic factors is limited but highly suggestive *(Bouchard, 1991).* To date more than 120 candidate genes have been related with obesity *(Razquin et al., 2011).* Among these genes some of the monogenic forms have come forward such as fat mass and obesity associated gene (FTO), leptin and melanocortin receptor 4 (MC4R). Sonestedt *et al.,* reported that among

22,779 individuals FTO genotype was significantly associated with both fat mass and lean mass and their data exhibited a further interaction between leisure time, physical activity and FTO genotype on cardiovascular mortality *(Sonestedt et al., 2010).* In FTO knockout mice, energy expenditure was shown to be increased whereas overexpression of FTO led to a dose-dependent increase in body weight and fat mass regardless of standard chow diet or high-fat diet *(Church et al., 2010).* In a different study, the association between FTO polymorphism and serum leptin concentrations were examined in European adolescents. The minor A allele of FTO was found to be significantly related to higher serum leptin concentrations independent of adiposity *(Labayen et al., 2010).* Therefore, it was concluded that leptin could be a possible intermediary contributing to the relationship between FTO and adiposity. The leptin receptor gene polymorphism was also shown to play an important role in development of obesity *(Riestra et al., 2010).* Leptin receptor R109K polymorphism was shown to correlate with obesity along with sweet preference *(Einosuke et al., 2008).* Rare heterozygous mutations in the coding sequence of MC4R was found to be associated with severe obesity cases in a Dutch population *(van den Berg et al., 2010).* A similar relationship was noted in a group of genotyped 796 individuals who undertook a 12 week resistance training program to test the response of BMI and subcutaneous fat *(Orkunoglu-Suer et al., 2010).* The results showed that females with a copy of the rare allele for MC4R had significantly higher BMIs and exercise in females did not alter MC4R expression. The genetic contribution to the development of obesity has been estimated at 40-70% *(Loos, 2009).* However, twin studies

have revealed that genetic variation associated with obesity is less common in those with a higher level of physical activity *(Silventoinen, 2009, Graff et al., 2010)*. It is clear that there are inherited differences in the susceptibility to obesity under given environmental and life style conditions.

1.1.3 Complications of obesity

Numerous epidemiological studies have shown that the risks associated with weight gain include the development of diabetes, heart disease, stroke, high blood pressure, and cancers, together with physical disability *(Lake et al, 2010)*. Table 1.2 lists the approximate relative risks for the complications.

Table 1. 2 Approximate relative risk of complications associated with obesity

Relative risk >3	**Relative risk 2-3**	**Relative risk 1-2**
Type 2 diabetes	Coronory heart disease	Cancer
Gallbladder disease	Hypertension	Reproductive hormone abnormalities
Dyslipidemia	Osteoarthritis	Polycystic ovary syndrome
Insulin resistance	Hyperuricemia	Impaired fertility
Breathlessness	Gout	Low back pain
Sleep apnea		

(World Cancer Research Fund and American Institute for Cancer Research Food, Nutrition, Physical Activity, and the Prevention of Cancer, 2007)

Type 2 diabetes is one of the most important disorders that have been associated with obesity. The induction of insulin resistance by obesity is thought to be the major cause of type 2 diabetes. A meta –analysis of 89

studies, which was conducted to provide an estimate of the incidence of each co-morbidity related to obesity, showed that elevated BMI and waist circumference had the strongest association with incidence of type 2 diabetes *(Guh et al., 2009).* In an another analysis of health burdens associated with overweight and obesity, the prevalence rate of type 2 diabetes was 3 to 4 fold greater in overweight and obese subjects *(Must et al., 1999).* It is estimated that 60-90 % of individuals who have type 2 diabetes are obese *(Mantzoros, 2006).*

Obesity is recognised as a risk factor for a number of cardiovascular risks including hypertension, dyslipidemia and coronary heart diseases *(Garrison et al., 1996, Lavie et al., 2009).* In the Multi-Ethnic Study of Atherosclerosis, it was found that higher BMI was associated with greater prevalence of adverse levels of blood pressure, lipoproteins and higher prevalence of hypertension in 6814 subjects aged between 45-84 years *(Burke et al., 2008).* In a different cohort study, indices of abdominal obesity (as defined by waist circumference or waist to hip ratio), but not BMI, were found to be consistently and strongly predictive of coronary heart disease *(Canoy et al., 2007).* It is not known whether fat accumulation in any specific tissue is more strongly predictive of morbidity and mortality than adiposity in general *(Stanner,2005).* However, there is evidence that visceral (also known as central or upper-body) obesity is more detrimental than peripheral (also known as limb or lower-body) obesity. Visceral fat accumulation has been

linked to increasing the flux of non-esterified fatty acids to the liver *(Stanner, 2005)*

The association between obesity and cancer has been suggested in a number of studies *(Gerhardsson et al., 1990, Morimoto et al., 2002, Giovanucci, 2003).* Although the findings are controversial in this area, it has been suggested that there was convincing evidence that obesity increased the risk of cancers of the esophagus, pancreas, colon and rectum, breast (post-menopausal), endometrium and kidney *(World Cancer Research Fund and American Institute for Cancer Research, 2007)*

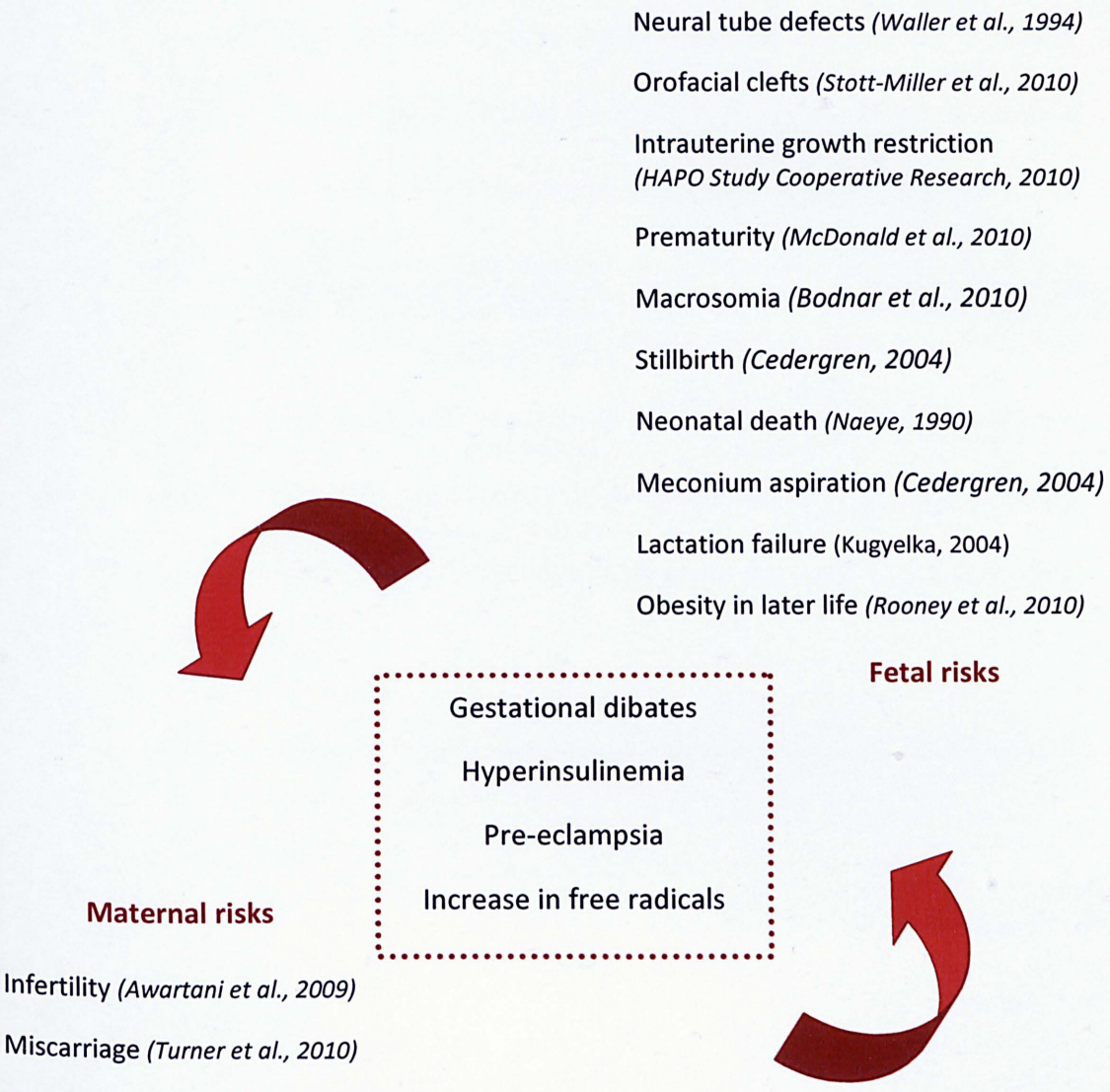
A large body of evidence has linked obesity to other health consequences such as respiratory diseases *(Piper and Grunstein, 2007),* chronic kidney diseases *(Ting et al., 2009),* musculoskeletal disorders *(Wearing et al., 2006),* gastrointestinal and hepatic disorders *(Batty et al., 2008 and Tsai et al., 2004)*, physical functioning and performance *(Woo et al., 2007)* and psychological problems *(Puder and Munsch, 2010).*

1.2 Maternal Obesity and It's Complications

The Health Survey for England during 2004 revealed a serious increase in the prevalence of obesity among women from 16.4 % in 1993 to 23.8 % in 2004 (Krishnamoorthy et al., 2006). More specifically, 13 % of 21- to 30-year-old and 22 % of 31- to 40-year-old females in England were found to be obese in 2007. It was estimated that this would rise to 30 and 47 % respectively by 2050 *(World Health Organization, 2000).*

The increasing proportions of obesity for women of child bearing age indicate the importance of the problem. Many of the pathological changes that are associated with the metabolic syndrome have been reported in obese pregnant women, including elevated fasting insulin concentrations, impaired endothelial function, elevated blood pressure, and increased pro-inflammatory markers *(Dereure et al., 1995, Samuels-Kalow et al., 2007).* To some extent these complications apply also in the non-pregnant state and majority of obese mothers may have normal pregnancies without complications. However, even moderate degrees of obesity have been found to be associated with several major risks to the fetus. These include structural abnormalities and greater perinatal mortality (Figure 1.1). The mechanisms involved in the development of these abnormalities are not clearly understood but it has been suggested that gestational diabetes, preeclampsia and increase of production of free radicals may be the key factors *(Beucher et al., 2010, Salihu et al., 2011, Sattar et al., 1996).*

Figure 1. 1 Complications associated with maternal obesity



1.2.1 Maternal Risks Associated with Maternal Obesity

During pregnancy, the energy requirement of the mother increases due to the increase in metabolic rate by 10-15 % *(Yu et al., 2006)*. According to FAO (Food and Agriculture Organization) and WHO recommendations, this additional energy should be 300 kcal per day. In contrast to past years, the dramatic increase in obesity has prompted changes to weight gain protocols for pregnant women. These are based on the pre-pregnancy weight of women in the United States (Table 1.3) *(Krishnamoorthy et al., 2006)*. In the UK, no specific recommendations have been given for weight gain according to BMI at conception.

Table 1. 3 Recommended total weight gain for pregnant women depending on their pre-pregnancy BMI

Pre-pregnancy BMI	**Recommended weight gain (kg)**
Underweight, <19.8	12.5-18
Normal, 19.8-24.9	11.5-16
Overweight, 25-29.9	7-11.5
Obese, > 29.9	6.8-9

From the beginning of pregnancy, the mother's body rapidly undergoes physiological and biochemical changes. Firstly, the total blood volume of the mother increases as a result of the increase in red blood cell mass and plasma volume *(Ward et al., 2007)*. This covers the increased oxygen demand. The increased oxygen demand also leads to other cardiovascular adaptations such as elevation of cardiac output and heart rate *(Ward et al., 2007)*. It has been suggested that maternal obesity may impact upon cardiovascular adaptations adversely during this stage. Every 100 g fat

deposited increases the cardiac output by 30-50 mL/min *(Vasan, 2003).* Pressure overload triggers sympathetic activity through leptin, insulin and various inflammatory mediators. Throughout these systems, obesity-induced alterations can have profound effects on cardiac, endothelial and vascular functions during pregnancy *(Davies, 2007).* As a result, maternal hemodynamic changes in obese mothers lead to higher arterial blood pressure, hemoconcentration and altered cardiac function compared to normal weight pregnant women *(Galtier-Dereure, 2000).*

Pre-eclampsia is a hypertensive disorder of human pregnancy. It is diagnosed on the basis of increased blood pressure (when it is over or equal to 140/90 mmHg) and proteinuria (when it is over or equal to 300 mg/24 h). It is one of the leading causes of maternal death with risks of convulsion, kidney and liver failure. It occurs in 5-7% of all pregnancies *(Walsh, 2007)* and is the main cause of preterm delivery *(Lydakis et al., 2011).* According to data from various studies based on hypertension and pre-eclampsia during pregnancy, obese women are at greater risk for hypertension (between a range of 2.2-21.4 times) and for pre-eclampsia (1.22-9.7 times) than control groups *(Galtier-Dereure 1995).* The pathophysiology of pre-eclampsia is based on an imbalance between thromboxane and prostacyclin, which causes activation of circulating leukocytes and vascular cell dysfunction through vasoconstrictor mechanisms. Oxidative stress and inflammatory markers like C-reactive protein, inflammatory cytokines, tumor necrosis factor-α (TNF- α),

interleukin-6 (IL-6) and interleukin-8 (IL-8) are also elevated in pre-eclampsia *(Walsh, 2004, Qiu et al., 2004, Kauma et al., 2002).*

In a study of the risk of hypertensive disorders by BMI and age-adjusted hazard models, 13,722 women were screened. Compared with the reference category of normal weight it was shown that overweight women showed nearly a 3-fold greater risk, and obese women had more than a 5-fold excess risk of hypertensive disorders of pregnancy *(Samuels-Kalow et al., 2007).* Moreover, pre-pregnancy BMI was also shown to be related to the occurence of hypertensive disorders.

During pregnancy, major adjustments occur in maternal carbohydrate and lipid metabolism in order to minimize protein catabolism. As glucose is the preferred fuel for the fetus, maternal hepatic glycogen stores are mobilized and hepatic glucose production increases *(Harding, 2001).* Even in the fed state, maternal blood glucose levels remain elevated for longer periods *(Harding, 2001).* These natural outcomes of pregnancy may develop into Gestational Diabetes Mellitus (GDM), with maternal obesity. GDM is glucose intolerance first recognised in pregnancy *(Yu et al, 2006).* The usual increase in insulin resistance seen in late pregnancy to maximize glucose availability for fetus is enhanced in obese mothers *(King, 2006).* GDM increases risk of adverse pregnancy and infant outcomes, and is also associated with increased risk of type 2 diabetes later in life in both mothers and children *(Chu et al., 2007).* A systematic meta-analysis showed that risk of developing GDM was 2 times higher in overweight, 4 times higher in obese

and 8 times higher in severely obese women when compared with normal weight pregnant women *(Chu et al., 2007).* 50% of women with a history of GDM develop diabetes within 5-10 years after delivery *(Chu et al., 2007).* GDM increases risk of adverse birth outcomes, especially if it is unrecognised and untreated *(Gillman et al., 2003).* GDM results in an increased risk of caserean delivery, postpartum hemorrhage and vaginal lacerations.

When considering the other antenatal risks associated with maternal obesity, thromboembolism (formation of a clot in a blood vessel that obstructs the flow of blood through the circulatory system) is one of the leading causes of maternal death *(Krishnamoorthy et al.,2006).* The prevalence of thromboembolism is 0.04% in normal weight women, but this proportion increases to 0.07% in overweight and 0.08% in obese women *(Yu et al., 2006).*

Maternal obesity is also shown to increase risk of many complications during intrapartum period. Caesarean delivery rates are consistently higher in obese women *(Kumari, 2001).* Each 1-unit increase in BMI elevates the risk of a caesarean section by 7% *(Galtier-Dereure et al., 1995).* Increased risk of induction of labour, failed instrumental delivery, hypoxic complications, post-partum haemorrhage, post-partum infections, increased length of hospital stay *(Krishnamoorthy et al., 2006).*

When compared with women who were of normal weight before pregnancy, women who were overweight showed a 42% increase in risk of

death and those women who were obese showed 143% increase *(Samuels-Kalow et al., 2007).*

1.2.2 Fetal Risks Associated with Maternal Obesity

Maternal obesity impacts upon fetal development during the embryonic period and later gestation. This has important effects on postnatal development of the child. Human pregnancy consists of 2 important stages: the embryonic period and the fetal growth-development period *(Harding, 2001).* During the embryonic period and implantation, placentation is incomplete and at that stage the embryo is nourished by oviductal and then uterine secretions *(King, 2006).* These uterine secretions are a rich source of growth factors *(Siega-Riz et al., 2006).* It was shown that in the presence of increased free radicals during pregnancy, these secretions were perturbated by free radicals and these conditions inhibited the proper formation and functioning of the placenta later on gestation *(Siega-Riz et al., 2006).* At the same time, adipose tissue, as an highly active endocrine organ, secretes metabolites, cytokines and growth factors which may impact on the embroyonic environment *(Waki et al., 2007).* When considering the important factors that explain the association between maternal obesity and increased congenital anomalies, it is consistently suggested that maternal adiposity alters development during the sensitive embryonic period and through to the end of gestation. Congenital malformations like neural tube defects, spina bifida, anencephaly, oral clefts, heart anomalies, hydrocephaly and abdominal wall abnormalities are all shown to be related to maternal

obesity *(Waller et al., 2005).* Maternal obesity was found to be associated with significantly increased risk for offspring with spina bifida, heart defects, limb reduction defects and diaphragmatic hernias with odds ratios ranging from 1.33 to 2.10 *(Waller et al., 2005).*

The mechanisms underpinning these observations are not clearly understood. One of the suggested mechanisms relates to undetected type 2 diabetes or GDM *(Salbaum and Kappen, 2010).* Moreover, an increase in free radical production alters the expression of transcription factors and may contribute to embryopathology *(Loeken, 2004).* For neural tube defects, deficiency of folic acid is still considered the main reason, but some studies also show poor glycemic control may be more important than folic acid levels in obese subjects *(Waller, 1994).* The failure in detection of these abnormalities due to the difficulty in sonographic assesment of obese women may also explain the higher prevalence of congenital defects.

Macrosomia (newborn with an excessive birth weight) is another adverse outcome of birth related with maternal obesity. Obese mothers deliver large-for-gestational age (LGA) infants 1.4-1.8 times more frequently than do lean mothers *(Galtier-Dereure et al., 2000).* More importantly, macrosomic children have long-term complications during their adult life, with increased risk of childhood obesity, followed by adult onset diseases such as obesity, hypertension, coronory heart disease and diabetes *(Vohr et al., 2008, Boney at al., 2005, Gillman et al., 2003).* Oken and colleagues examined the relationship between gestational weight gain and childhood

adiposity among 1044 mother-child pairs *(Oken et al., 2007).* Gestational weight gain was directly associated with child overweight at age of 3 years. Women with adequate or excessive gain had approximately a 4-fold greater risk of having an overweight child. In another study assesing the effects of maternal hyperglycemia on childhood obesity, 9,439 mother-child pairs were screened and the highest quartile of hyperglycemia was associated with a significantly higher level of childhood obesity when compared with the lowest quartile. Increasing maternal glycaemic level was associated with a greater prevalence of macrosomia *(Hillier et al., 2007).* Importantly, these results suggested that increased risk for childhood obesity with maternal hyperglycaemia was modifiable by treating GDM, as obesity risk was no longer significant after multivariate adjustment in treated GDM groups *(Hillier et al., 2007).*

Since the 1950s, with improvements in medical care, stillbirth and neonatal deaths have declined, but there are still cases occurring roughly one in every two hundred births *(Frets, 1990, Glinianaia et al., 2010).* The causes of almost one-half of stillbirths are still unknown. According to a meta-analysis based on maternal obesity and risk of stillbirth, the odds of a stillbirth were 1.47 and 2.07 higher among overweight and obese pregnant women *(Chu et al., 2007).* This implies that obese mothers are twice as likely to have stillbirth babies as normal weight mothers. Like congenital anomalies associated with maternal obesity, mechanisms linking maternal obesity to stillbirths are unidentified. Poor glycaemic control and undiagnosed diabetes

are considered as likely mechanisms *(Chu et al., 2007).* On the other hand, another study reporting that maternal obesity more than doubled the risk of stillbirth and neonatal death did not associate mortality, gestational diabetes and maternal hypertension *(Kristensen et al., 2005).* Other responsible mechanisms may relate to the physiologically adverse effects of maternal obesity, such as difficulties in medical treatment or apnea-hypoxia events and more episodes of oxygen desaturation during sleep, which can reduce blood flow to the fetus. Indeed, Kristensen *et al.,* observed a greater ratio of fetoplacental dysfunction which may be explained by impaired placental blood flow in obese women in their study. Maternal adiposity could also produce hypoxia through increased glycosylated hemoglobin levels and decreased O_2 affinity, which then causes reduced O_2 transfer to the uterus by impairing normal placentation *(Chu et al., 2007).*

Obesity in pregnancy is also associated with impairment of lactogenesis. High BMI values before conception are negatively associated with initiation and duration of breastfeeding *(Rasmussen et al., 2001).* This may imply that infants of obese women tend to be formula fed, which was shown by some studies to predict childhood obesity later in life *(Ong et al., 2006, Moreno et al., 2007).* It is complicated to address the specific mechanisms involved in impairment of lactogenesis in humans as the success of breast feeding is based on psychosocial and sociodemographic factors. However, a rat study examining lactogenesis in obese mothers revealed that plasma prolactin concentrations did not respond to lactation normally *(Shaw*

et al., 1997). There was a significant increase in control animals from day 18 of pregnancy to day 3 of lactation but obese mothers failed to exhibit this increase. These findings may suggest a physiological impairment of lactogenesis in obese mothers.

1.3 Developmental Origins of Health and Disease

1.3.1 Epidemiological Studies

The Developmental Origins of Health and Disease concept emerged from early epidemiological studies. In 1923 Kermack, introduced the idea of early life exposures and their influence on later disease by using the analyses of birth cohorts in Great Britain and Sweden *(Kermack et al, 1934).* Their data showed that life expectancy was determined by the conditions which existed during a child's early years and the health of the adult was determined importantly by the environmental constitution which the child had built up. This was pre-dated by other studies which were showing birth cohort influences on mortality rates (*Derrick, 1927; Davidson, 1927)* and post-dated by other studies that linked this idea to diseases in later life such as tuberculosis *(Frost, 1939).*

Barker reported a strong geographical relationship between ischemic heart disease mortality rates in 1968-1978 and infant mortality rates in 1921-1925 *(Barker, 1986).* Barker's second report tested this hypothesis on 5654 men born during 1911-1930 in six districts of Hertfordshire. Data from this cohort showed that men with the lowest birth weight had the highest coronary heart disease (CHD) death rate and men with the highest birth

weight had the lowest CHD death rate *(Barker, 1989).* A similar relationship was confirmed by the study of a Helsinki cohort born during 1934-1944 *(Eriksson et al, 2001).* Boys who had a low ponderal index (birth weight/length) at birth, were shown to have an increased risk of coronary heart disease if their body mass rose more rapidly in childhood *(Eriksson et al., 2001).* This led to the conclusion that a determinant of high risk ischemic heart disease could be the inadequate early life environment.

Data from the Hertfordshire cohort also showed that type 2 diabetes prevalence steadily increased with lower birth weight *(Barker 1993).* These findings led to the proposition that undernutrition during gestation reprogrammes the relationship between glucose and insulin and between growth hormone and insulin-like growth factors. These adaptations permanently change the body's structure, function and metabolism and increase risk for ischemic heart disease in later life *(Barker,1993).*

The Dutch Hunger Winter gives the unique opportunity to assess the adverse effects of early under nutrition on adulthood disease risk. During the Second World War, food transport from the rural east to the urban west of the Netherlands was limited due to the severe winter conditions and Nazi blockade *(de Rooji et al, 2006).* Therefore, general energy intake of the adult population declined to 1400 calories in October 1944 and to 400-800 calories in November 1944 *(Roseboom et al, 2001).* Although the children under 1 year old were protected from the famine, pregnant and lactating women could not be protected totally. After the liberation of the Netherlands in early

May 1945, food intakes improved quickly. The reports based on follow ups of the Dutch Hunger Winter babies showed not only the adverse effects of early life famine exposure on adulthood health status but also the importance of the timing of the exposure during fetal life. Glucose intolerance and impaired insulin secretion, increased obesity (Ravelli et al., 1999), altered lipid profile and cardiovascular diseases were associated with prenatal exposure to famine, especially for those exposed to the famine during early gestation *(Roseboom et al, 2001).* On the other hand, exposure to famine during mid-gestation was associated with reduced renal function *(Roseboom et al, 2001).* In addition to these effects, breast cancer, dysfunction of mental performance, schizophrenia, childlessness, early menopausal age were all identified as programmed outcomes in Dutch Famine cohort studies *(Roseboom et al, 2001).* However, no evidence was found for programming of increased blood pressure *(Roseboom et al, 2001).*

The siege of Leningrad (the German blockade of the city known as St Petersburg) between Sep 1941 and January 1944 prevented food supplies to the city and the average energy intake of the population declined to 300 calories with virtually no protein *(Stanner et al, 1997).* Average birth weights decreased by 18% in males and 16% in females during the starvation period *(Antonov, 1947).* 169 subjects exposed to malnutrition were compared with 188 subjects who were living outside the famine region (unexposed control group). Results showed that subjects exposed to malnutrition showed evidence of endothelial dysfunction and a stronger influence of obesity on

blood pressure but no association was observed between intrauterine malnutrition and glucose tolerance, insulin concentration, lipid concentration and blood pressure *(Stanner et al, 1997).*

One of the main criticisms of the epidemiological studies on developmental origins of health and disease is the data sets relying on the assessment of the environment in adult life *per se*. The necessity of detailed longitudinal assessment of the environment from before conception through pregnancy, fetal life, birth and infancy is crucial for these studies *(Collins, 2002).* Secondly, it has been suggested that in order to elucidate the interactive effects of genetics and early life environmental risk factors on chronic complex diseases such as hypertension, it becomes essential to assess the various risk factors simultaneously in the same cohort rather than in separate cohorts *(Collins, 2002).*

In addition to argumentative points of historical cohort studies, birth weight data was considered as an indirect measurement for the assessment of the growth of the human fetus since alterations in maternal diet are considered as less influential factors on birth weight when compared to genetical and socio-economical factors *(Campbell, 1991).* Evidence about the variation in maternal diets that influence birth weight is limited and often exhibited contradictory results *(Doyle, 1992, Mathews et al.,1999).* No relation was found between birth weights, head circumferences and maternal nutritional intakes during pregnancy of over 176 women in another study *(Langley-Evans and Langley-Evans, 2003).* It was shown that low birth weight

was mainly associated with lower socio-economic status during childhood and adolescence *(Bartley et al., 1994).*

Furthermore, in a meta-analysis of 55 studies, Huxley *et al* reported that the association between birth weight and subsequent blood pressure could be due to the impact of random error, selective emphasis of particular results and inappropriate adjustment for confounding factors *(Huxley et al., 2002).* Similarly, Kramer and Joseph reported inconsistent outcomes to Barkers' hypothesis *(Kramer and Joseph, 1996).* In a retrospective cohort study, systolic blood pressure of adults born at low birth weights was found to be similar to those with normal birth weights *(Matthes et al., 1994).*

1.3.2 Concepts of Fetal Programming

Several studies conducted to investigate the developmental origins of health and disease led to development of different sub-hypothesises about the underlying mechanisms of fetal programming. In this section the Thrifty Phenotype, Predictive Adaptive Response and Fetal Insulin Hypothesis will be explained.

The Thrifty Phenotype Hypothesis states that poor nutrition *in utero* leads to fetal adaptations that produce permanent changes in insulin and glucose metabolism *(Hales and Barker, 1992).* Under poor conditions the fetus would spare the growth of vital organs such as brain at the expense of the other tissues such as muscle and pancreas *(Hales and Barker, 1992).* This would occur by compromised development of pancreas, reduced β cell number and function that lead to permanent changes in the glucose-insulin

axis *(Freeman, 2009).* It was suggested that compromised glucose homeostasis would not be problematic to individuals who continue to be thin but in conditions of nutritional excess leading to insulin resistance, obesity, metabolic syndrome and type 2 diabetes this situation becomes detrimental *(Hales and Barker, 2001).*

The Predictive Adaptive Response (PAR) Hypothesis suggests that the fetus makes adaptations *in utero* or in the early postnatal development period based on the predicted postnatal environment *(Gluckman and Hanson, 2006).* When this PAR is appropriate, the adult phenotype is normal; however, where mismatch occurs between the predicted and actual environment, disease manifests *(Gluckman and Hanson, 2006).* The main difference of the PAR Hypothesis is that it creates a response to a given *in utero* or early postnatal nutritional environment either in high or low conditions. Moreover, the cellular processes that are adapted to cope with the predicted environment could be in favour of the survival phenotype depending on the postnatal environment. For instance, genes promoting the induction of insulin resistance in response to early fetal environment have been favoured in ancestral humans as they created a phenotype which would have improved survival under sub-optimal conditions *(Rickard and Lummaa, 2007)* Table 1.4 shows the survival phenotype traits and their proposed adaptive benefits *(Rickard and Lummaa, 2007).*

Table 1. 4 Survival phenotype traits and their proposed adaptive benefits

Trait	Possible adaptive value
Insulin resistance	Reduces energy invested into growth and metabolism
A preference to lay down visceral fat	Emergency fuel reserve, acting as a buffer in unpredictable conditions
Reduced skeletal muscle	Decreases energetic demands of maintaining adult body and frees up resources for survival and reproduction
Reduced capillary density in some tissues	Reduced postnatal food availability equates to less to less exchange of nutrients and hormones between organs and tissues
Reduced nephron number	Appropriate to smaller body size and smaller kidneys
Reduced negative feedback in the hypothalamus-adrenal axis	Heightened stress response allows greater chance of survival in a nutrient deprived and therefore predator rich environment

(Rickard and Lummaa, 2007)

Therefore, it has been proposed that negative consequences of the survival phenotype could be observed only when the adult environment offers an abundant food source *(Gluckman et al, 2005).* This point of view gains more importance when considering societies undergoing rapid industrialisation which now show a rapid increase in metabolic syndrome and type 2 diabetes incidences *(Gluckman and Hanson, 2005).*

Fetal insulin secretion plays an important role in fetal growth *(Milner and Hill, 1987).* The Fetal Insulin Hypothesis suggests that insulin mediated fetal growth will be affected by genetic factors that regulate either fetal

insulin secretion, or the sensitivity of fetal tissues to the effects of insulin *(Hattersley et al., 1999).* It has been reported that macrosomic babies born to women with diabetes in pregnancy did not overgrow as a result of a direct increase in the transfer of nutrients. Macrosomia was mediated indirectly by increased fetal insulin secretion in response to fetal sensing of maternal hyperglycaemia *(Metzger et al, 2009).*

1.3.3 Animal Studies

The limitations of epidemiological studies, such as ethical reasons and complex nature of human life, promoted the animal models for investigating nutritional programming of adult diseases. In order to elucidate the mechanisms underpinning the programming of the fetus, various animal models using different species and dietary interventions have been developed. Target diseases include hypertension *(Langley Evans et al., 1996),* insulin resistance *(Simmons et al., 2001),* impaired glucose homeostasis *(Boloker et al., 2002),* obesity *(Kirk et al., 2009),* atherosclerosis *(Yates et al., 2009),* renal dysfunction *(Bursztyn et al., 2006),* susceptibility to oxidative damage (Franco et al., 2002), cancer *(Polanco et al., 2010)* and impaired immunity *(Lockwood et al., 1988).* Dietary manipulations based upon restriction of macro- or micro-nutrient intakes such as protein *(Langley and Jackson, 1994),* iron *(Lewis et al., 2001),* magnesium *(Venu et al., 2005),* zinc *(Tomat et al., 2010)* and overall food intake *(Woodall et al., 1996),* and over-nutrition of macro- or micro-nutrient intakes such as fat *(Armitage et al., 2005),* sugar *(Frazier et al., 200)*and sodium *(Porter et al., 2007)* have been

used in different studies. A variety of large (sheep, pig) and small (mouse, rat, guinea-pig) animal species have been used to test hypotheses and improve the understanding of developmental origins of diseases *(McMullen and Mostyn, 2009).*

1.3.4 Low Protein Diet Models

One of the first developed and most widely reported animal model for investigations of programming is the low protein dietary rat model *(Langley-Evans, 2001).* Generally in such studies, pregnant rats are given isocaloric diets which include 5-9 % casein compared with 18-20 % casein in control groups. When considered relative to the normal rat protein requirement, 5% casein diets are extremely low but 8-9 % casein diets may be classified as mild restricted diets. In order to match the energy components of control and low protein groups the carbohydrate content is increased by addition of starch and sucrose with a proportion of 2:1 *(Langley-Evans, 2004).*

To date low protein diets in rodent pregnancy have been associated with several disorders in adults such as hypertension (Langley-Evans et al., 1999), diabetes *(Barnett et al., 2008)* and obesity *(Bellinger et al., 2005, Breier et al., 2001).* The exact mechanisms leading to these disorders are not clearly understood yet. However, altered tissue development, endocrine functions and epigenetic changes are thought to be responsible *(Gong et al., 2010, Theys et al., 2009, Langley-Evans et al., 1996).*

Nutrient utilization by fetal tissues is one of the most important processes for organ development and functioning of the fetus. Rat fetuses

exposed to maternal low protein diets show lower cell numbers in some trunk organs like kidney *(Hoppe et al., 2007)* and pancreas *(Chamson-Reig et al., 2006)* cells compared to control groups. These reductions in the cell numbers of organs may account for impaired organ functioning at later life. It has been suggested that reduced maternal protein intake supresses the renin-angiotensin system (RAS) of the newborn and that this impaired intrarenal RAS then causes permenant alterations in the formation, structure and later function of the kidneys. This may explain the decreased level of glomerular volume and surface area and lower glomerular filtration rate *(Ingelfinger et al., 2002, Woods et al.,2004).* Altered tissue development may also drive the progression of diabetes. Increased β-cell apoptosis, decreased proliferation and modified islet cell expansion, leads to a smaller endocrine β-cell mass *(Fowden et al., 2005, Reusens et al., 2006).* Furthermore, initial increased insulin responses to a glucose challenge are followed by reduced insulin secretion and degranulation of islets, suggesting that the secretory capacity is readily exceeded in offspring of protein restricted animals.

1.3.4.1 Mechanisms of programming in low protein model

Glucocorticoids, mineralocorticoids, androgens and oestrogens are the steroid hormones produced from the adrenal cortices and gonads *(Laycock and Wise, 1983).* They are typically associated with long-term organizational effects on homoestasis throughout the body. Glucocorticoids exert their effects on several metabolic pathways and have important functions in brain development, glucose and blood pressure homeostasis, maturation of trunk

organs like lung, heart, kidney, gut and as well as regulating gene expression *(Harding, 2001).* Because of these functions, glucocorticoids appear to be important influences on development during fetal life. Under normal circumstances fetal levels of glucocorticoids are much lower than maternal levels due to the placental enzyme 11 ß-HSD-2 (11 ß-hydroxysteroid dehydrogenase type 2) which acts as a barrier to maternal corticosteroids *(Seckl, 2004).* The main function of 11 ß-HSD-2 is the conversion of corticosterone to 11 dehydro corticosterone through maternal to fetal environment in rats and consequently provides much lower circulating levels of active glucocorticoid for the fetus. Relative deficiency of 11 ß-HSD-2 is shown to increase glucocorticoid exposure of the fetus *(Edwards et al., 1996).* This results in retarded fetal growth and maladapted developmental processes that may increase the risk of chronic disease through adult life *(Seckl, 2004).* Some of the studies based on the effects of dietary protein restriction on glucocorticoid and 11 ß-HSD-2 homeostasis show that maternal low protein (MLP) diet during rat pregnancy selectively modifies activity and expression of 11 ß-HSD-2 *(Langley-Evans et al., 1996).* Offspring of rats which experience glucocorticoid treatment during pregnancy show low birth weight and become hypertensive *(Drake et al., 2007).* Moreover, maternal diet-induced hypertension would appear to be a glucocorticoid-dependent phenomenon. Treatment of pregnant protein restricted rats with an inhibitor of glucocorticoid synthesis, completely prevented hypertension development in the offspring *(Langley-Evans et al, 1997).* Conversely, inhibition of 11 ß-

HSD-2 in pregnancy induced hypertension in offspring of rats fed a control diet.

Liver is the major trunk organ and is primarily important for carbohydrate homeostasis and blood glucose regulation. As discussed above glucocorticoids also have essential hepatic functions through modifying carbohydrate metabolism. One of the key enzymes of carbohydrate metabolism, phosphoenol pyruvate carboxykinase (PEPCK), is stimulated by glucagon to increase gluconeogenesis by producing phosphoenolpyruvate. This is shown to be affected by antenatal glucocorticoids *(Nyirenda et al., 2001).* Rats that are exposed to excess glucocorticoid in utero demonstrate increases in expression of PEPCK as adults *(Gicquel et al., 2008).* In a study of rats exposed to MLP in utero, glucokinase activity was shown to decrease (approximately 50%), whereas PEPCK activity increased (approximately 100%), in the low-protein and recuperated offspring compared with controls at 21 days of *age (Desai et al., 1997).* Over-expression of PEPCK leads to increased gluconeogenesis and opposes the inhibitory effects of insulin on gluconeogenesis. This is thought to be a risk factor for glucose intolerance in later life by means of inducing fasting hyperglycemia, reactive hyperglycemia and hyperinsulinemia *(Meaney et al., 2007).* Type 2 diabetes programming by low protein diets may also be driven by altered receptor- hormone function in insulin sensitive tissues like liver, muscle and adipocytes *(Godfrey et al., 2006).* These tissues are shown to have increased insulin receptor number, altered insulin receptor substrate 1 and 2 associated phosphotidylinositol 3-

kinase (PI3K) and selective insulin resistance in rats exposed to protein restriction in utero. GLUT-4, which mediates the action of insulin on glucose uptake, has also been shown to be increased in such animals *(Ozanne et al., 2003 and Gluckman et al., 2004).*

Methylation pathway alterations in protein restricted models are another candidate mechanism for fetal programming of adult diseases. It is known that low protein diets can induce changes in DNA methylation, which may affect gene transcription, receptor expression and may also drive transgenerational effects because of the inheritability of epigenetic changes *(Gluckman et al., 2004 and Harrison et al., 2009).* Permanent alterations to the phenotype of offspring subject to maternal undernutrition suggest that fetal growth retardation is associated with stable changes in gene expression *(Simmons, 2007).* When considering the importance of methylation related factors for tissue modelling like purine synthesis, tissue growth, DNA methylation, histone acetylation and gene expression, this point of view gains more importance. Protein restriction in pregnant rats induces persistent loss of DNA methylation and greater expression of some hepatic genes (glucocorticoid receptor) in the offspring *(Gicquel et al., 2008).* Owing to these alterations, perturbation of methylation pathways by low protein diets is suggested as a programming mechanism for blood pressure and glucose intolerance. Moreover, methylation is an element of single carbon metabolism involving other micronutrients like the glycine-serine cycle, B_6 and B_{12} vitamins *(Gluckman et al., 2006).* In pregnant rats fed a low-protein diet,

supplementation of the dam with glycine or folate prevents hypertension, endothelial dysfunction and vascular dysfunction or reduces endothelial nitric oxide synthase expression in the offspring, respectively (*Torrens et al., 2006, Brawley et al., 2004).* It has also been suggested that periconceptional and early pregnancy nutrient-gene interactions, related to folate, link vascular related reproductive complications and cardiovascular diseases in adulthood *(Steegers-Theunissen et al., 2003).* Aberrant changes in the DNA methylation of genes important for fetal programming, such as the glucocorticoid receptor gene or the insulin like growth factor-2 (IGF2) gene, are caused by protein restriction, and some of these changes can also be prevented by dietary supplementation with cofactors *(Gicquel et al., 2008).* However, it was shown that altered DNA methylation in maternal low protein diet was not related to major perturbations of folate metabolism since maternal and fetal tissues in this study did not exhibit difference in the expression of folate and one-carbon metabolism intermediates *(Engeham et al., 2010).* This hypothesis was also examined by investigating the glucokinase gene in rat offspring programmed using a maternal low protein diet *(Bogderina et al., 2004).* Results showed no significant disturbance in expression and promoter areas of glucokinase gene. It was concluded that maternal low protein diet may exert its effects on methylation patterns at a distance from the promoter or factors which regulate basal glucokinase expression.

One concept central to the Thrifty Phenotype Hypothesis is that, because of nutritional thrift, an undernourished fetus may be more prone to

obesity in conditions of adequate or overnutrition *(Breier et al., 2001).* According to behavioral studies on animal models using the MLP diet, offspring show altered behaviours toward feeding and locomotor activity *(McMillen et al., 2005).* It was shown that in young adult rats, early life exposure to undernutrition determines high preference for fatty foods *(Bellinger et al., 2004).* However, in a further study which aimed to assess the long-term impact of undernutrition during fetal life, it was suggested that fetal undernutrition does not programme obesity in rats without postnatal dietary challenge *(Bellinger et al., 2006).* The basis of this phenomenon is not completely understood, but altered hypothalamic-pituitary-adrenal axis (HPA) function is shown as a potential target *(Matthews, 2002).* This may include some programming of hypothalamic hormones and derivatives, as well as long-term appetite control and meal-related peptide hormones such as hippocampal glucocorticoid receptors, brain-derived neurotrophic factors, corticotropin-releasing hormone, neuropeptide-Y, leptin, ghrelin and cholecystokinin *(Grove et al., 2005).*

1.3.5 High Fat Diet Models

Obesity is a worldwide epidemic that is characterized by excess adipose tissue. This contributes to numerous chronic diseases and early mortality *(Racette et al., 2003).* It is known that the aetiology of obesity is multi-factorial and composed of genetic, behavioural, environmental, physiological, social, and cultural factors that result in energy imbalance and promote excessive fat deposition. However, it is still not clear how these

factors interact with each other. Obesity and related disorders are generally attributed to environmental factors, including overnutrition driven by high energy and high fat content foods. At the same time, there is now accumulating evidence of programming of obesity, appetite regulation and body composition during the fetal life of individuals *(Zambrano et al., 2006 and Lopes et al., 2008).* However, these studies have predominantly focused on maternal undernutrition and the influence of maternal overnutrition and obesity on fetal development remains largely uncharacterised. In addition to this, the most frequently studied obese animals, those with genetic or hypothalamic obesity, or diet-induced obesity generated by offering free access to diets with high palatability and/or high fat content, are relatively infertile *(Rolls and Rowe, 1982 and Rolls et al., 1986).* Previous studies have shown that 67 % of diet-induced obese rats became pregnant whereas this proportion was 86 % for the normal weight control rats (*Rolls et al., 1986).* Guo *et al.,* observed a significant decrease in reproductive performance of rats receiving a 23% fat diet when compared to control animals which were receiving 4% fat *(Guo et al., 1995).* Maternal behavioural disorder leading to cannibalism and energetic inhibition of reproduction were reported as possible mechanisms of the relatively poor reproductive performance *(Glick et al., 1990, Rolls and Rowe, 1982).* It was shown that maternal diabetes together with 30 % fat feeding led to higher mortality rates among the offspring, in addition to maternal reproductive disabilities *(Koukkou et al., 1998).* Surviving offspring from those litters exhibited decelerated growth rates during suckling which may suggest failure of lactation associated with

high fat diet *(Poston and Taylor, 2007).* At the time of weaning these offspring were shown to have impaired vascular function when assessed in mesenteric arteries. Since a considerable number of obese rats do become pregnant, it has been possible to determine how obesity affects maternal adaptation to pregnancy, fetal development and later health status of the resulting offsprings.

1.3.5.1 Cafeteria Diet Induced Obesity in Rats

The cafeteria diet protocol first developed by Rothwell and Stock *(Rothwell and Stock, 1979)* involves offering laboratory animals *ad libitum* access to an assortment of energy rich foods processed for human consumption *(Rogers and Blundell, 1984).* This has been shown to result in marked increases in voluntary food intake and rates of weight gain relative to rats fed a standard maintenance diet *(Rogers and Blundell, 1984).* Another advantage of this model is that the interpretation of the causes of or potential cures for obesity is not complicated by physiological or behavioural abnormalities *(Llado et al., 1995).* Moreover, because of their high content of calories, fat (mainly saturated), salt and refined sugars and low content of vitamins and essential nutrients, cafeteria diets are thought to be a useful experimental model for reflecting the adverse effects of the western high-fat and high-sugar diet which is considered as a primary risk factor for developing obesity in humans *(Bayol et al., 2007, Macqueen et al., 2007).*

Despite the advantageous effects of cafeteria diet on inducing obesity in laboratory animals, some drawback points were discussed in literature,

previously. Firstly, lack of standardization between the cafeteria diets used in different studies was considered as an important point since the macro- and micro-nutrient composition can not be controlled *(Moore, 1987).* Secondly, it was reported that accurate measurements of energy intake were difficult to achieve as it was possible for each animal to prefer an individual selection of foods that were offered which may result in variation between the animals *(Reuter, 2007).* Also, overestimation of food consumption due to water evaporation of the foods was reported *(Shafat et al., 2008).* However, this effect was shown to be minimal *(Akyol et al., 2009).* It was also concluded that these criticisms can be overcome by careful use of the feeding protocols and well-controlled experiments *(Rothwell and Stock, 1988).*

The mechanisms through which cafeteria diet fed rats are influenced by these heavily processed, highly palatable and hyperenergetic foods are not clear and the suggestion that hyperphagia-inducing diets are more palatable than laboratory chow diets alone is thought not to fully explain the observed effects. Hyperphagia, altered feeding behavior, appetite regulation and food variety are the main concerns in this regard. When meal frequency, meal duration, mean meal size and eating rate were measured in cafeteria diet fed rats versus controls *(Rogers and Blundell, 1984),* both palatability and variety of foods were found to be important factors in determining the degree of hyperphagia. More importantly the level of adiposity was found to be dependent on the structure of feeding behaviour. Meal frequency was reduced as a consequence of the development of obesity. This pattern of

infrequent feeding was also suggested as a factor in the etiology of human obesity.

The excess weight gain of cafeteria diet fed rats was found to be due mainly to the accumulation of additional body fat instead of change in the lean body mass *(Rogers and Blundell, 1984).* Biochemical responses of cafeteria fed animals also change, resulting in lowered energy efficiency and active thermogenesis, low plasma levels and hepatic production of urea and low tissue capabilities for amino acid catabolism and nitrogen handling *(Llado et al., 1995).* Further in vivo measurements of metabolic alterations in cafeteria diet fed rats showed that, because cafeteria diet contains a high proportion of fat (approximately 50% of total energy), most of the triglyceride deposited was derived directly from the diet, and the energy cost of this process is lower (approximately 5 kJ/g). As a result fat synthesis from carbohydrate is reduced by about 50% in cafeteria fed rats compared to animals eating a relatively low fat diet (9% of total energy) *(Rothwell et al., 1982).* On the other hand, it was suggested that the main cause of obesity in these animals is suppression of brown adipose tissue thermogenesis which can be seen in all animal models of obesity *(Roca et al., 1999).*

It is well known that adaptive thermogenesis is of critical importance for energy expenditure in rodents. Brown adipose tissue thermogenesis largely depends on the activity of the uncoupling protein 1 (UCP1) and the main physiological regulator is noradrenaline which innervates the tissue, promotes brown adipocyte cell proliferation and differentiation through UCP1

activation and synthesis *(Verty et al., 2010, Bartness et al., 2010,).* There is some evidence of altered UCP1 activity in cafeteria diet fed rats *(Rothwell et al., 1982 and Roca et al., 1999).* It has been suggested that cafeteria diet fed rats increase both lipolytic activity in white adipose tissue and UCP expression in brown adipose tissue (UCP1) and muscle (UCP3) to avoid weight gain *(Rodriguez et al., 2004).*

The response to cafeteria diet induced overfeeding has shown to be sex-dependent with respect to higher weight gain, defective brown adipose tissue thermogenesis abd expression of key adipogenic transcription factors *(Rodriguez et al., 2001).* In a study of the short term effects (2 weeks) of cafeteria diet feeding in both male and female rats, the increase in visceral fat (95% increase) was found to be more sensitive to cafeteria diet induced hypertrophy than the increase in subcutaneous fat (33% increase for inguinal white adipose tissue and 25% for inguinal brown adipose tissue) *(Rodriguez et al., 2004).* The difference was thought to be due to a differential pattern of expression of several adipogenic transcription factors, especially peroxisome proliferator-activated receptor (PPAR)-γ2 *(Rodriguez et al., 2004).* At the same time, greater hypertrophy of gonadal white adipose tissue was found in females, whereas males showed a greater hypertrophy of inguinal white adipose tissue. This gender differential response was also associated with the mRNA expression of PPAR-γ2.

In addition to differences between genders, diet and the duration of diet induced adiposity are thought to be important factors determining gene

expression in adipose tissue samples *(Al-Hasani and Joost, 2005).* The expression of some important genes implicated in lipid metabolism were shown to be up-regulated, whereas other genes related to redox and stress were down-regulated following 8 weeks cafeteria diet feeding in Wistar obese rats *(Lopez et al., 2003).* Specifically, expression levels of glycerol-3-phosphate dehydrogenase, stearoyl CoA desaturase, fatty acid binding protein (FABP), uncoupling protein-3 (UCP3) together with transcription factors (CAAT/enhancer binding protein-α and PPARγ) were found to be increased in these animals, indicating enhanced lipogenesis. All of these results may suggest that cafeteria diet feeding is a useful method to induce obesity by producing changes in expression of transcription factors and their downstream targets.

Metabolic biomarkers in cafeteria diet fed rats have also been shown to be affected deleteriously. In a study of rats in which two different obesogenic experimental dietary models (1 high in saturated fatty acids by supplementing chow diet with 10% beef tallow, 2 cafeteria diet prepared from chocolate cake mix, carrots and eggs) were used from weaning, liver morphology and function was assessed against controls *(MacQueen et al., 2007).* Liver morphology and function were altered in animals eating a cafeteria diet (being large and steatotic), while the high fat and the control rats did not differ significantly. The livers of cafeteria rats were found to be so packed with glycogen and lipid that the cellular and tissue structures appeared to be disrupted in these animals.

1.3.5.2 The Effect of Maternal Cafeteria Diet/High Fat Feeding on Rodent Offspring

As in humans, in rats of normal body weight, maternal body fat stores increase during pregnancy and continuously higher levels of glucose utilization by the fetus results in maternal hyperphagia and progressive alterations of glucose metabolism *(Rolls and Rowe, 1982 and Rolls et al., 1986).* Postabsorptive hypoglycemia and an insulin resistant state are the main consequences of this alteration. For the high energy demand of lactation, further fat is deposited during gestation. This temporary state during pregnancy is considered to be similar to the metabolic profile of high-fat feeding. From these common points between pregnancy and diet induced obesity or overweight, arise the questions of how fetal development and adult disease risk of offspring are affected by the additive load of maternal adverse metabolic conditions induced by overnutrition.

Although the relationships between birth weight and disease risk later in life show inconsistencies in the epidemiological studies, for most of the nutrient restricted animal models a plausible causal association has been demonstrated *(Langley-Evans, 2004).* However, there is relatively little work considering associations between maternal overfeeding and later disease. The available literature is sometimes contradictory. One of the earliest reports on this subject showed that, although pregnancy in the cafeteria diet fed rats appeared to proceed normally and litter number did not differ between the groups, the mean birth weight of the pups was slightly reduced *(Rolls and*

Rowe, 1982). After birth, the pups of control mothers gained weight more rapidly and by the first week of lactation the mean pup weights in the obese group were significantly lighter than those of control mothers. One of the possible causes of this unexpected result was thought to be the lower protein intake of the obese mothers and a lack of essential micronutrients, whereas the chow diet was nutritionally balanced for these nutrients.

In contrast, cafeteria diet feeding in pregnancy was also shown to be a model for gestational diabetes mellitus, which generally results in macrosomia *(Holemans et al., 2004).* However, in cafeteria diet fed mothers glucose tolerance was impaired with a rise in insulin. Litter weight was found to be increased because of an increased litter size. Furthermore, it was shown that because of the immaturity of the newborn rat, macrosomia in rats was difficult to induce.

More specifically, Bayol *et al* examined the effects of a maternal cafeteria diet on skeletal muscle and adipose tissue development in the offspring of three different experimental groups which were fed cafeteria diet throughout gestation and lactation (CDW) or cafeteria diet during gestation and standard chow diet during lactation (CDG) or only standard chow diet *(Bayol et al., 2005).* According to these results, the three diets did not influence birth weights and postnatal growth rates but the CDW pups exhibited a markedly increased adiposity at weaning, characterised both by an increase in fat pad weight and intramuscular lipid deposition. It is well known that intramyocellular fat accumulation disrupts normal muscle insulin

sensitivity and therefore this is accepted as a reliable marker of whole body insulin resistance. Here, the CDW pups also exhibited an increased intramyocellular fat accumulation with an attempt to maintain normal insulin sensitivity by increasing levels of insulin like growth factor-1 (IGF-1), IGF-1 receptor and PPARγ mRNA. This suggested that these pups had an increased propensity for insulin resistance *(Bayol et al., 2005).* Although the increased adiposity was prevented in the CDG offspring, muscle atrophy and fibre hypoplasia remained. This situation shows that defective muscle structure may remain into adulthood as a detrimental effect of maternal overfeeding.

In order to examine the feeding behaviour and food preference of pregnant and lactating rats and the influence of a maternal cafeteria diet style feeding (junk foods) on appetite regulation, food preference, body weight gain and activity levels in the rat offspring, a study was conducted with six groups of rats which were fed either rodent chow alone or with junk food diet (biscuits, marshmallows, cheese, jam doughnuts, chocolate chip muffins, butter flapjacks, potato crips and caramel/chocolate bars) during gestation, lactation and/or post-weaning *(Bayol et al., 2007).* Rats offered the junk food diet consumed 40% more food and 56% more energy than control rats during pregnancy, with only 20% of the total energy consumed throughout pregnancy originating from the rodent chow. The rats kept on the junk food diet during gestation continued to be hyperphagic and increased the intake of foods rich in fat, sucrose and salt. However their body mass was comparable to control groups.

The offspring born to mothers fed the cafeteria diet exhibited significantly lower birth weights and switching from the junk food diet during gestation to chow diet alone during lactation was found to be more detrimental for the growth of the offspring, as the pups from this group were lighter than the other groups. More importantly, rats exposed to the junk food diet during gestation and lactation exhibited 18% and 26% daily increase in energy intake compared to other offspring. Further analysis showed that when the junk food diet was offered during the post-weaning period, an increased preference for junk food was characterised by a selective preference for foods rich in fat, sugar and salt (but not protein and fibres). The mechanisms through which the cafeteria diet programmed appetite change and fat gain were not identified. The lactation stage may be of importance. It has also been shown that high fat feeding or cafeteria diet feeding may alter the milk composition of rats in previous studies *(Rolls and Rowe, 1982, Rolls et al., 1986).*

Khan *et al.* reported impaired endothelium dysfunction and relaxation in the pups of control mothers which were suckled by fat-fed dams *(Khan et al., 2005).* In addition to this, there is also evidence that feeding a diet rich in lard to pregnant rats leads to gender-related cardiovascular dysfunction in normally fed offspring *(Khan et al., 2003).*

Similar patterns of programming of hyperphagia, adiposity and increased adult disease risk factors have been shown in different models of diet induced obesity in rodents *(Samuelsson et al., 2008, Srinivasan et al.,*

2006 and Buckley et al., 2005). In one study, 100 day old female C57BL/6J mice were fed either a standard chow diet or a semi-synthetic energy-rich and highly palatable obesogenic diet (10 % simple sugars, 20 % animal lard, 28 % polysaccharide, 23 % protein) for 6 weeks before mating. The mice were maintained on the same diets throughout gestation and suckling *(Samuelsson et al., 2008).* All pups were then weaned onto standard chow. Energy intake and weight gain were significantly greater in dams fed the obesogenic diet compared with chow fed controls and by day eighteen of gestation they were hyperinsulinaemic and hyperleptinaemic, although maternal weights converged at the end of gestation. The offspring from obese mothers exhibited significantly elevated plasma triglyceride, insulin and leptin concentrations, systolic blood pressure, diastolic blood pressure (for males only) and increased reactivity to noradrenaline at 3 months. At the same time, adipocyte hypertrophy in these animals was evident in inguinal fat tissue. Adipose gene expression data showed that PPARγ type2 expression was significantly increased, whereas, mRNA expression of ß-adrenoceptor 2 and 3 and 11-ßHSD type 1 were significantly reduced in the offspring from obese mothers. Hypothalamic energy balance regulatory center, locomotor behaviour and adipocyte metabolism were all thought to be affected adversely by maternal high fat feeding.

In all of these studies, both maternal obesity and maternal high fat diets were described as contributing factors to programming of offspring while the individual effects of these factors have not been assessed broadly.

Recently, an overfeeding based model of maternal obesity in rats using intragastric feeding of diets via total enteral nutrition tested this hypothesis by feeding two groups of dams with control or 15 % energy increased diets for 3 weeks before mating *(Shankar et al., 2008)*. At the time of mating the group which were fed by energy dense diet had significantly heavier body fats and this was due to higher adiposity. Following mating all of the groups were fed by the obesogenic diet throughout the pregnancy. Results showed that offspring of obese dams gained significantly higher body weight and had higher body fat percentage when fed a high-fat diet. Therefore, it was concluded that maternal body composition at conception itself had important implications for offspring adiposity. Another difference of this study was that the energy dense diet was formed with a higher degree of carbohydrate (75 %) rather than fat (5 %) in the treatment group.

In a different study, the separated effects of maternal obesity and maternal diet on resulting offspring were assessed by using three study groups which were low fat (LF) , high fat (HF) and high fat but pair fed to the caloric intake of low fat group (PF) 4 weeks before mating, during pregnancy and lactation *(White et al., 2008)*. In this study, the group PF represented the effects of high fat diet during pregnancy only. At 18 weeks of age offspring of HF dams weighed the most, exhibited hyperleptinemia and insulin resistance whereas offspring of LF and PF dams exhibited similar results. In parallel with Shankar et al., this study indicated that maternal obesity was necessary to induce the adverse effects of programming of obesity.

These studies suggest that the effects of maternal adiposity and high fat diet during pregnancy may induce differentiated effects on adult offspring. Since most of the studies examining the programmed fetus of overfed and obese mothers have largely failed to define the individual consequences of these effects, it is necessary to establish more detailed study designs which may reveal further explanation.

1.4 Molecular Mechanisms of Insulin Resistance

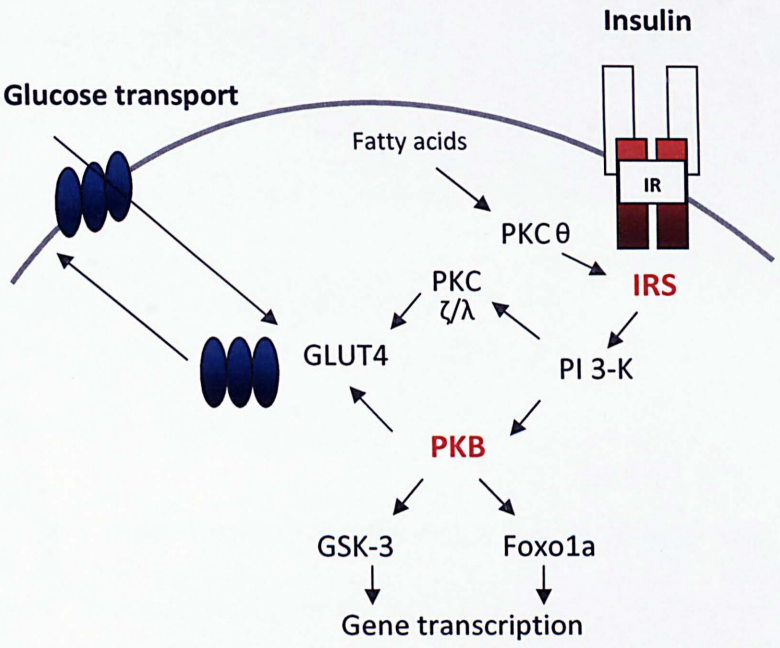
As discussed in Section 1.1.3 type 2 diabetes is the most common endocrine disorder associated with obesity. Currently, it affects over 170 million people in world-wide and it is estim ated that this figure will be over 365 million by the year 2030 *(Wild et al., 2004).*

Type 2 diabetes is characterized by high blood glucose, insulin resistance and relative insulin deficiency (Fryn, 2003). The main pathophysiology of type 2 diabetes relates to β-cell failure of the pancreas. In addition to this, resistance of target tissues to insulin is an important cofactor contributing to the development of type 2 diabetes. The main role of insulin is to lower blood glucose concentrations by facilitating glucose uptake mainly into skeletal muscle and fat tissue, while inhibiting endogenous glucose production by the liver. Insulin resistance results from complex interactions between overnutrition, excess fatty acid intake, oxidative stress and hypoxia of the adipose tissue *(Tanti and Jager, 2009).* Under insulin resistant conditions, the insulin related organs do not function properly and consequently plasma glucose concentrations remain high. At the same time,

insulin secretion from pancreas increases to compensate the higher concentrations of plasma glucose. This creates a vicious cycle which leads to the dysfunction of β-cells and developing peripheral insulin resistance *(Khan, 2003).* Although the background of the pathophysiology of type 2 diabetes is well known, gene targeting approaches and naturally occurring mutations in animal models have contributed to a deeper understanding of the molecular dysregulation of type 2 diabetes.

The insulin signalling pathway, where insulin elicits series of signalling events to maintain normal plasma glucose concentrations, is a critical component of overall energy homeostasis *(Zhang et al., 2009).* However, the signalling pathways which mediate the metabolic actions of insulin remain largely unclear. There is a growing body of evidence which is implicating that the insulin activated, phosphatidylinositol 3' –kinase (PI 3K)-dependent Ser/Thr kinase Akt as a regulator of glucose transport, glycolysis, protein synthesis, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival determination of cell size and cell-cycle progression *(Whiteman et al., 2002).* Figure 1.2 represents the insulin signalling pathway.

Figure 1. 2 Schematic representation of insulin signalling



Binding of insulin to the IR activates PI 3-K through IRS. Downstream of PI 3-K, e PDK mediates activation of PKB. Activated PKB can regulate transcription of target genes via GSK-3 or Foco1a. Also, PKB regulates glucose uptake by recruiting GLUT-4 to the plasma membrane. Downstream of the IR the signalling cascade branches with PKC as additional downstream effectors of insulin. Modulation of PKC θ activity by fatty acids may impair signalling through IRS/PI 3 kinase and GLUT4 dependent glucose transport. IR: Insulin receptor, IRS: Insulin receptor substrate, PDK: Phosphoinositide-dependent kinase, PI 3-K: Phosphatidylinositol 3-kinase, PKB: Protein kinase B, PKC: Protein kinase C, GSK-3: Glykogen synthase kinase-3, GLUT4: Glucose transporter 4, Foxo: Forkhead box protein.

1.4.1 Insulin Receptor Substrate (IRS) Proteins

IRS was first identified by antiphosphotyrosine immunoprecipitation as a 185-kDa tyrosol-phosphorylated protein (pp185) in insulin stimulated cells *(White et al., 1985).* Several IRS isoforms were subsequently found in almost all tissues and cells *(Sun and Liu, 2009).* IRS1 and 2 were found to be the most important IRS proteins in the regulation of carbohydrate

metabolism *(White, 2002).* Rare mutations of the IRS1 are associated with insulin resistance in humans *(Yamauchi et al., 1996).* IRS1 knockout in mice results in insulin resistance mainly of muscle and fat. In addition to this, mice missing this gene developed phenotypes similar to insulin receptor knock-out mice, including significantly smaller body size, impaired glucose tolerance and impaired insulin-stimulated glucose uptake in muscle *(Araki et al., 1994, Tamemoto et al., 1994).*

IRS2 expression has been shown in liver, heart, adipose tissue, brain, pancreas and muscles *(Sun et al., 1997).* IRS-2 knockout mice develop diabetes as a result of β-cell failure *(Previs et al., 2000).* In ob/ob and db/db mice (recognized type 2 diabetic models with hyperinsulinemia, hyperglcemia and hyperlipidemia) IRS2 levels are down-regulated *(Zhang et al., 2009).* On the other hand, in the type 1 diabetic rodent model of hypoinsulinemia, IRS2 mRNA expression was significantly increased (Shimomura et al., 2000).

1.4.2 AKT/Protein Kinase B Proteins

Protein Kinase B (AKT) proteins are serine/threonine protein kinases that are thought to play a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration *(Whiteman et al., 2002).* Insulin-dependent AKT proteins have been shown to mediate the effects of insulin on glucose transport, glycogen synthesis, protein synthesis, lipogenesis and suppression of hepatic gluconeogenesis *(Whiteman et al., 2002).* Akt regulates both glucose uptake via facilitated glucose transporters (GLUTs) and intracellular glucose

metabolism in insulin sensitive tissues such as muscle. However, studies that assess the function of AKT in normal and diabetic patients found controversial results as the function of PKB was shown to be impaired in muscle and adipocytes of diabetic patients *(Krook et al., 1998 and Rondinone et al., 1999).* In contrast a different study reported no impairment of AKT activity in muscles of diabetic patients *(Kim et al., 1999).*

AKT knockout animal models offer clearer understanding of the roles of AKT proteins in glucose homeostasis. It has been shown that disruption of AKT1 in mice did not result in any significant perturbation in metabolism *(Cho et al., 2001, a).* On the other hand, AKT2 (PKBβ) knockout mice showed insulin resistance, resulting in a phenotype of impaired insulin-mediated glucose disposal and lack of suppression of hepatic glucose production in response to insulin *(Cho et al., 2001, b).*

1.5 Aims and Hypotheses

1.5.1 Aims

The aim of this thesis was to create a novel study design to examine the individual and/or combined effects of excess maternal adiposity and maternal cafeteria diet feeding on:

i. Maternal adaptation to pregnancy, maternal body composition and metabolism
ii. Fetal growth and development
iii. Nutritional intakes, body composition and metabolical biomarkers of offspring at 13 weeks of age
iv. Glucose homeostasis of offspring at 13 weeks of age

1.5.2 Hypotheses

The hypotheses to be explored in this thesis are:

i. The feeding of maternal cafeteria diet or maternal obesity will result in profound obesity, disturbed glucose and lipid metabolism of mothers during pregnancy.
ii. The feeding of maternal cafeteria diet or maternal obesity will impair fetal growth and development.
iii. The feeding of maternal cafeteria diet or maternal obesity will programme obesity, hyperphagia, increased adiposity, disturbed lipid metabolism and glucose intolerance in offspring at 13 weeks of age.

2.0 MATERIALS and METHODS

2.1 Animal Procedures

Animal experiments described in this thesis were performed under licence from the Home Office in accordance with the 1986 Animals (Scientific Procedures) Act. Each of the animal trials discussed in this report used rats of the Wistar strain purchased from Harlan (UK). Rats were housed in plastic cages and subjected to a 12-hour light/dark cycle at a temperature of 20-22°C. The rats had *ad libitum* access to food and water at all times. Experimental animal procedures are outlined below.

2.1.1 Cafeteria Diet

A cafeteria diet comprises offering laboratory animals highly energetic and highly palatable foods to trigger diet induced obesity. The cafeteria diet in this study included strawberry jam, short bread biscuits, potato crisps, fruit-nut chocolate, Mars bars, roasted and salted peanuts, golden syrup cake, pork pie, cocktail sausages, liver and bacon pate and cheese. Cafeteria diet fed rats were given four of these palatable foods randomly, in excess quantities each day and two of them were changed every day in order to maintain variety. They were also provided with chow diet *ad libitum*. Each of the foods including the chow was weighed before and one day after they were offered to rats. Nutritional values of the palatable foods were calculated from manufacturers' information (Appendix, Table 7.1). Weight loss due to

evaporation was measured in triplicate samples of each individual food item placed in empty cages. The average daily percentage change in the weight of foods ranged from 0 to 6.2% and corresponded to an average overestimation of energy intake by 2.51% (7.5 Kj/day). This amount was considered as an acceptable error of measurement.

2.1.2 Pilot Trial

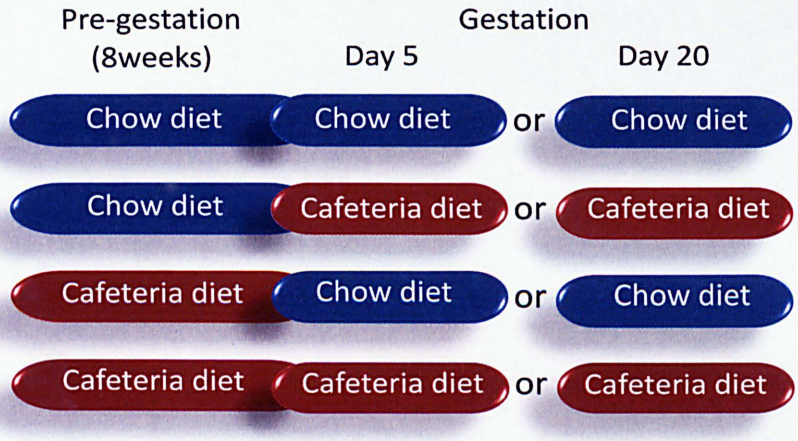
At the beginning of the experiments a pilot trial was conducted to assess whether maternal obesity affected the reproductive abilities of the rats. In this context, Virgin female Wistar rats (aged 3 weeks, n=8) (Harlan LTD, Belton, Leics, UK) were randomly allocated to be fed either a control chow diet alone (C, n=4) (B&K Universal Limited, UK) (Appendix Table 7.2) or a control chow diet alongside a cafeteria diet (O, n=4) for six weeks as described in 2.1.1. Rats were then mated with Wistar stud males and kept on the same pre-gestational diets until day 5 of pregnancy. On day 5 of pregnancy, determination of plasma volume procedure was performed (as described in 2.2.4). Maternal gonadal fat, peri-renal fat, intrascapular fat (brown and white) and liver were accurately sampled, weighed and stored at -80°C. Carcasses were retained to determine body composition (Section 2.2.6). All of the animals and their food intake were weighed and recorded daily.

2.1.3 Maternal Trial

Virgin female Wistar rats (aged 3 weeks, n=47) (Harlan LTD, Belton, Leics, UK) were randomly allocated to be fed either a control chow diet alone, or a control chow diet alongside a cafeteria diet as described in section 2.1.1.

After eight weeks of control or cafeteria diet feeding, all rats were paired with a Wistar stud male. Mating was confirmed by the appearance of a semen plug on the cage floor. In order to separate the effects of maternal obesity from high-fat diet *per se,* half of the animals from the control group were switched to cafeteria diet (CO, n=12) and half of the animals from the cafeteria group were switched to a control diet (OC, n=11) at conception. The remaining animals within each group were maintained on their pre-gestational diets (CC, n=12 and OO, n=12). To assess the effects of cafeteria diet in early and late gestation, half of the animals from each of the final four groups were culled on day 5 and the other half on day 20 of gestation, after plasma volume measurement (as described in 2.2.4). Maternal gonadal fat, peri-renal fat, intrascapular fat (brown and white), liver and kidneys were accurately weighed and sampled and stored at -80°C until required for further analysis. Carcasses were kept to determine body composition (Section 2.2.6). All of the animals and their food intake were weighed and recorded daily.

Figure 2 1 Study design of maternal trial



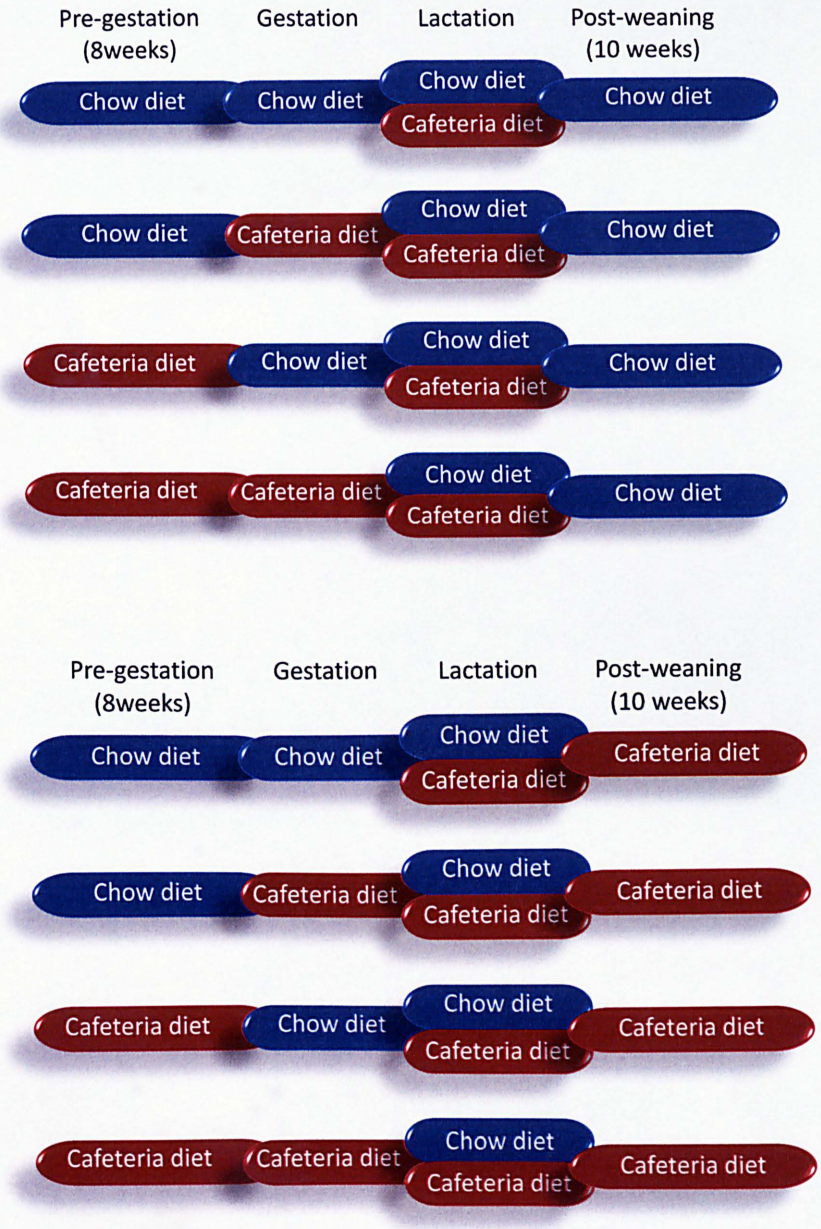
2.1.4 Offspring Trial

Virgin female Wistar rats (aged 3 weeks, n=64) (Harlan LTD, Belton, Leics, UK) were randomly allocated to be fed either a control chow diet alone (C, n=29) (Teklad Global 18% Protein Rodent Diet, UK) (Appendix, Table 7.3) or a control chow diet alongside a cafeteria diet (O, n=35) (as described in 2.1.1). After eight weeks of control or cafeteria diet feeding, all rats were paired with a Wistar stud male. Mating was confirmed by the appearance of a semen plug on the floor of the cage. In order to separate the effects of maternal obesity from high-fat diet *per se,* half of the animals from the control group were switched to cafeteria diet (CO, n=18) and half of the animals from the cafeteria group were switched to a control diet (OC, n=20) at conception. The remaining animals within each group were maintained on their pre-gestational diets (CC, n=11 and OO, n=15). Maternal body weight and food intake were recorded daily.

At birth, all litters were culled to maximum of 8 pups within 12 hours after delivery. This minimised variation in nutrition during lactation period. Since it has been shown that the cafeteria diet feeding during lactation alone may induce adverse programming effects on rats (Bayol *et al*, 2007), each of the four maternal groups were divided into two to be fed by chow diet or cafeteria diet during lactation. This created eight different study groups at the end of the lactation. Offspring body weights were measured within 12 hours after delivery, on postnatal day 7, postnatal day 14 and on the day of weaning (day 21). Maternal food intake was weighed twice in a week.

Due to the evidence that the postnatal environment is also important for the development of the adverse effects of programming *(Bellinger et al., 2004)*, an additional nutritional challenge was added to the study at the time of weaning. At 4 weeks of age three male and three female offspring from each litter were weaned onto standard chow diet and housed in groups, whereas one male and one female offspring were weaned onto cafeteria diet and housed individually. All animals and their food intake were weighed twice a week. At 13 weeks of age all animals were culled following an intraperitoneal glucose tolerance test (section 2.2.5). Gonadal fat, perirenal fat, liver, brain, left kidney and right kidney were accurately weighed and then stored at -80°C until used for further analysis. Carcasses of the animals which had the cafeteria diet during post-weaning period were kept for further body composition analysis.

Figure 2 2 Study design of offspring trial



2.2 Chemical Analysis

All chemicals and reagents were purchased from Sigma-Aldrich (UK), unless otherwise stated.

2.2.1 Coefficient of Variation (COV)

Intra-assay coefficient of variation was calculated to compare readings from duplicate samples from the same assay. Inter-assay coefficient of variation was calculated to compare readings from the same sample from different assay procedures.

COV= Standard Deviation / Mean x 100%

These measures indicate variability and reliability within each of the chemical/biochemical assays.

2.2.2 Preparation of Plasma

Blood samples were collected into heparinised tubes/capillaries, and initially left to stand on ice. They were then centrifuged at 13,000 x g, 4°C for 15 minutes. Plasma was removed and stored at -80°C until further analysis.

2.2.3 Determination of Circulating Metabolites

2.2.3.1 Glucose Assay (non-fasted and fasted)

Plasma glucose concentration was determined using an adaptation of the glucose oxidase method (Trinder, 1969). For the measurement of maternal plasma glucose concentrations, baseline blood samples collected at the beginning of the plasma volume procedure were used (Section 2.2.4). For

the measurement of offspring plasma glucose concentrations blood samples collected during the intraperitoneal glucose tolerance test were used for fasted baseline and all subsequent time points. All samples and reagents were allowed to reach room temperature prior to analysis. A standard curve was constructed by making serial dilutions of glucose standard (0-2 µg glucose). Samples were diluted 1:5 with phosphate buffer. Standards and diluted samples were loaded in duplicate onto 96-well plate in 10 µl quantities and 200 µl of glucose reagent (prepared using 100 ml of 0.1 M sodium phosphate buffer, pH:7.4 and adding 5 mg glucose oxidase, 1 mg horseradish peroxidise and 0.1 g 2.2' azino-di-3[ethyl-benzthiazolin-sulphonate]) was added to each well. The plate was then incubated at 37°C for 15 minutes. Absorbance was then read at 620 nm using a Tecan Sunrise plate reader (Magellan Version 4.0 computer package). The inter- and intra-assay coefficients of variations were 2.8 % and 3.7 % respectively.

2.2.3.2 Cholesterol Assay

Plasma cholesterol concentration was determined using a commercial kit (Thermo Life Sciences, UK) following the manufacturer's instructions. For the measurement of maternal and offspring plasma cholesterol concentrations, baseline blood samples collected at the beginning of the plasma volume procedure (2.2.4) and blood samples collected at 120 minutes of the intraperitoneal glucose tolerance test were used. A standard curve was constructed by making serial dilutions of cholesterol stock (0-7.760 mmol standard). Samples were diluted 1:5 with water. 10 µl of either diluted

standards or samples were loaded in duplicate onto 96-well plate. 200 µl of cholesterol assay reagent was added and the plate incubated at 37°C for 15 minutes. The absorbance was then read at 550 nm using a Tecan, Sunrise plate reader (Magellan Version 4.0 computer package). The inter- and intra-assay coefficients of variations were 2.1 % and 3.2 % respectively.

2.2.3.3 Triglyceride Assay

Plasma triglyceride concentration was determined using a commercial kit (Thermo Life Sciences, UK) following the manufacturer's instructions. For the measurement of maternal and offspring plasma triglyceride concentrations, baseline blood samples collected at the beginning of plasma volume procedure (Section 2.2.4) and blood samples collected at 120 minutes of the intraperitoneal glucose tolerance test were used. A standard curve was constructed by making serial dilutions of triglyceride stock (0-2.280 mmol standard). Samples were diluted 1:5 with water. 10 µl of either diluted standards or samples in were loaded in duplicate onto a 96 well plate. 200 µl of triglyceride assay reagent was added and the plate incubated at 37 °C for 15 minutes. The absorbance was then read at 550 nm using a Tecan Sunrise plate reader (Magellan Version 4.0 computer package). The inter- and intra-assay coefficients of variations were 2.6 % and 4.3 % respectively.

2.2.3.4 Insulin Assay (non-fasted and fasted)

Offspring plasma insulin concentration was determined using an Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem Inc, USA) following the manufacturer's instructions. All reagents and samples were allowed to reach

room temperature prior to analysis. 95 µl of sample diluent was added to each well before 5µl of sample or standard was added to each well in duplicate. Then the plate was covered and allowed to incubate for two hours at 4°C. The plate was washed five times with 200 µl washing buffer, removing any remaining solution after each wash. 100 µl of rat insulin enzyme conjugate was added to each well and the plate was covered and incubated at room temperature for 30 minutes. Excess solution was removed by a further washing step (seven times with 200 µl washing buffer). Immediately, 100 µl enzyme substrate solution was dispensed and plate was left at room temperature for 10 minutes. For the baseline fasting samples this period was 40 minutes. During the enzyme reaction, the microplate was not exposed to light. The reaction was stopped by adding 100 µl of enzyme reaction stop solution and absorbance was measured within 30 minutes at 450 nm using a Tecan Sunrise plate reader (Magellan Version 4.0 computer package). Mean concentrations were calculated from the duplicated raw data quantified from the extended rat insulin standard curve (range 1-64 ng/ml for non-fasted samples and 0.1-12.8 ng/ml for fasted baseline samples) which included eight points using serial dilutions. The inter- and intra-assay coefficients of variations were 2.4 % and 4.8 % respectively.

2.2.4 Determination of Plasma Volume

Maternal plasma volume expansion was measured during day 5 and day 20 of gestation using the Evans Blue Dye Method *(Blair and Mickelsen, 2006).* Rats were terminally anesthetised using isoflurane. A cannula was

inserted into the left iliac vein and a baseline blood sample removed prior to administration of 0.3 ml Evans blue dye (0.5 mg/ml). After five minutes, a second blood sample was removed. Animals were euthanized by injection of pentobarbitone and death confirmed by cervical dislocation. Baseline and five minute blood samples were centrifuged at 13,000 rpm for 15 minutes and the absorbance of plasma samples determined at a wavelength of 610 nm. A standard curve was constructed by linear regression analysis of absorbance values obtained from known concentrations of Evans blue dye (0.001-0.15 mg/ml). Plasma volume was measured by calculating the dilution of the dyeThe inter- and intra-assay coefficients of variations were 1.9 % and 3.4 % respectively.

2.2.5 Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance tests were performed on offspring at the age of 13 weeks. All of the food was removed from cages 18 hours prior to the start of testing. This ensured that the test was done under fasted conditions. Each animal was weighed at the time when the food was withdrawn and at the time of the test. After restraining the animal, a blood sample was obtained from the lateral tail vein under local anaesthesia. This blood sample was designated as baseline. Within 5 minutes of sampling 1 ml/100 g body weight glucose (20 g/100 ml in 0.9% saline) was administered via the intraperitoneal route. This gave an overall dose of 2 g glucose/kg body weight. Further blood samples were taken at 5 minutes, 15 minutes, 30 minutes, 60 minutes. Animals were returned to their home cage between

sampling times. At 120 minutes animals were culled and blood samples were collected via a heart puncture. All samples were centrifuged at 13,000 rpm for 15 minutes and stored at -80°C for 6 months. Area under the curve data for glucose was performed using GraphPad Prism Version 5.

2.2.6 Body Composition Analysis

Maternal and offspring carcass composition were determined in samples of dried, homogenised rats as described in previous studies (Langley and York, 1990). Whole rat carcasses were individually weighed and placed in tin foil containers where they were dried out in a drying oven at 80°C. The rats were taken out and weighed every three to four days, and when the weight remained constant they were considered fully depleted of water and removed from the oven. Body water was determined from the difference between wet and dry weight of the carcass. The carcasses were then ground up and homogenised using a food processor, aliquotted into air tight plastic containers and stored at -20°C until further analysis.

2.2.6.1 Fat Extraction

The fat content of each sample was determined via the rapid Soxhlet extraction method using the Gerhardt Soxtherm. The Gerhardt Soxtherm heats a known amount of a sample until it gets dry. In this way fat is removed from the sample into previously weighed glass flasks. The difference between the initial and final weight of the flask gives the weight of the extract which can be used to obtain the total Soxhlet fat value per sample.

Each sample was weighed (approximately 2 g) onto an 11 cm qualitative grade filter paper, loosely folded to fit down to the bottom of the Soxhlet thimble and covered with a plug of cotton wool. Glass flasks, which were oven-dried and cooled in desiccator, were weighed with three anti-bump stones. Samples covered with a plug of cotton wool were placed in a flask. Flasks were then placed in the Soxtherm machine and extracted with 155 ml petroleum ether at a temperature of 150°C for 50 minutes. After completion of the programme flasks were placed in the oven at 100°C for 1 hour without the thimbles. Then flasks were transferred to a desiccator to cool down to room temperature and weighed.

2.2.6.2 Determination of Nitrogen

The nitrogen content of each sample was determined by using the micro-Kjeldahl method (Markham, 1942). The method uses a series of reactions to determine the nitrogen value of each unknown sample. The reactions progress as follows:

1. $(NH_4)_2 SO_4 + 2NaOH \longrightarrow Na_2SO_4 + 2NH_3 + 2H_2O$
2. $NH_3 + H_2O \longrightarrow NH_4OH$
3. $NH_4OH + HCl \longrightarrow NH_4Cl + H_2O$

1000 ml M HCL = 14 g Nitrogen

Using 0.2 M HCl, 1 ml 0.2 M HCl = 0.0028 g Nitrogen

0.5 g of sample was weighed onto filter paper. This was folded into a small package and placed into a labelled Kjheldal tube. 1 Kjeldahltab and 10 mls concentrated sulphuric acid were added to each tube. Sample tubes were lowered onto a digestion block (set at 400°C). After 50 minutes of digestion, samples were cooled for 20 minutes. The Vapodest apparatus was used to distil the samples. Samples were run in batches; each batch included standards and blanks. The amount and molarity of hydrocholoric acid was checked for titrations and the pH electrode was calibrated by carrying out a test run using distilled water. Tubes were placed singly on the Vapodest and the following procedure was carried out automatically.

1. 50 mls of distilled water and 50 mls NaOH (40 % w/v)
2. Steam distillation for 4 minutes into 50 mls of saturated boric acid
3. Titration of the ammonium hydroxide collected in the boric acid with dilute HCl
4. Percentage nitrogen is calculated and recorded

The percentage of nitrogen in each sample was calculated using the following equation:

% Nitrogen in sample = (Titre (minus blank)) x 0.0028 x 100) / Sample weight (g)

By knowing the nitrogen content in a sample it is possible to gain a value of protein content of any given sample by multiplying the nitrogen value by 6.25.

2.3 Analysis of Gene Expression

2.3.1 RNA Extraction and Sample Preparation

Offspring livers were sampled at time of cull and stored at -80°C for 12 months. Total RNA was isolated from frozen liver samples using the Trizol method (Invitrogen,UK). The RNA was treated with DNase (Promega,UK) and subjected to phenol-chloroform extraction and ethanol precipitation. RNA concentration was determined spectrophotometically and RNA integrity was confirmed on a 1% agarose gel electrophoresis.

2.3.2 Trizol Extraction

This method extracts RNA from small amounts of tissue. Approximately 100 mg of tissue was weighed into autoclaved 15 ml glass tubes and 1 ml Trizol reagent (Invitrogen, UK) was added. Tissue was then homogenised as quickly as possible using homogeniser, which was rinsed between each sample. The homogenate was pipetted into eppendorf tubes, centrifuged at 12,000 x g for 10 minutes at 4°C and supernatant was transferred to a fresh tube. After incubating the tube for 5 minutes at room temperature, 0.2 ml choloform was added and the tube was shaken vigorously by hand for 15 seconds. After incubating for a further 3 minutes at room temperature, samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. The upper, colourless, aqueous phase was transferred to a fresh tube and mixed with 0.25 ml isopropanol followed by 0.25 ml of a high salt precipitation solution (0.8 M $NaH_2C_6H_5O_7$ and 1.2 M NaCl). Samples were incubated for 10 minutes at room temperature before centrifuging at 12,000 x g for 10 minutes at 4°C.

Supernatant was discarded and then the RNA pellet was washed with 75% ethanol by vortexing.

2.3.3 DNase Treatment

RNA was treated with DNase to ensure that any remaining DNA contamination, which could affect results during later analysis, was removed. Each RNA sample in ethanol was centrifuged at 13,000 x g for 15 minutes at 4°C. After removing ethanol, samples were centrifuged for further a 3 minutes to ensure that all of the ethanol was removed. Then the RNA pellet was dissolved in 40µl RNase-free water, 5 µl DNase 10x buffer (Promega,UK) and 5 µl DNase (Promega,UK) respectively. Samples were then incubated at 37°C for 30 minutes after vortexing and spinning down. 150 µl RNase-free water and 200 µl phenol/chloroform/isoamyl alcohol (25:24:1, Fisher,UK) were added and then the samples were centrifuged at 13,000 x g for 5 minutes at 4°C. The top layer was removed, transferred to a fresh tube and 15 µl 3M sodium acetate (pH=5.5) and 375 µl 100% ethanol were added. At this point samples were stored at -80°C overnight for precipitation. When samples became glutinous and gloopy but not completely fluid after defrosting, they were centrifuged at 13,000 x g for 15 minutes at 4°C. Supernatant was removed, 1 ml 75% ethanol was added to wash the RNA pellet which was then stored at -80°C until further analysis.

2.3.4 Quantification of RNA Extracted

Extracted RNA stored in 75% ethanol was defrosted and centrifuged at 7500 x g for 5 minutes at 4°C. Ethanol was then removed, the RNA pellet was air dried and dissolved in 20 µl RNase-free water by passing through a pipette tip several times. RNA concentration was quantified using a Nanodrop spectrophotometer (Thermo, USA). 1.5 µl of each sample was placed onto the Nanodrop pedestal and measured in duplicate after using RNase-free water as a blank. Sample concentration, 260/280 and 260/230 ratio values were recorded. From this information the RNA content of the samples were standardised to 100 ng/µl by adding RNase-free water. The ratio of 260/280 and 260/230 was used to check that the RNA was of good quality and not degraded or contaminated. Ideally the ratio should be between 1.7 and 2.0, and samples below 1.5 were not used. The samples were then stored at -80°C until they were required for further analysis.

2.3.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to detect the presence and the level of insulin substrate 2 (IRS-2) and protein kinase B (Akt-2) in RNA samples extracted from offspring liver samples. This was done in a two-step method so that both the reverse transcriptase and the polymerase chain reaction could be optimised independently.

2.3.5.1 Reverse Transcriptase Reaction

The reverse transcriptase (RT) reaction synthesises complementary strand DNA. RNA which had previously been cleaned and precipated was used for the RT reaction at a concentration of 0.1 µg/µl. 5 µl of total RNA was mixed with 1 µl of random primers and 9µl RNase-free water in a 96-well PCR plate (ABgene). In a PCR machine (Applied Biosystems, UK) the plate was then incubated at 70°C for 5 minutes and then at 4°C while each of the following was added into each reaction well: 5 µl MMLV Reverse Transcriptase buffer x5 (Promega, UK), 1.25 µl Nucleotides (10mM each) (Promega, UK), 0.5 µl RNase inhibitor (Promega, UK), 1 µl MMLV reverse transcriptase (Promega, UK) and 2.25 µl RNase free water. The plate was returned to the PCR machine and incubated at 42°C for 60 minutes after incubating at room temperature for 10 minutes. As a control for contamination from genomic DNA and non-mRNA transcript the above reaction was carried out on a different plate for all samples without the MMLV enzyme. After the incubation each reaction was diluted in 1:4 with RNAse free water to give 100 µl cDNA stock. The total 100 µl volume was stored at -20°C prior to amplification by polymerase chain reaction.

2.3.5.2 Design of Primers

Primer sets were required for IRS2 and AKT2. Gene sequences for these genes were found using the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Accession numbers can be found in Table 2.1 along with forward and reverse primer sequences.

Primer sets were designed using Primer Express™ software version 1.5 (Applied Biosystems, UK). Primers were supplied by MWG Biotech, (Germany).

Table 2. 1 Primer sequences

Gene	Accession number	Forward Primer (5' →3')	Reverse Primer (5' →3')
IRS2	NM_001168633	CAAGAACCTGACCGGTGTATACC	GGCTGTTCGCAATTGAGCTT
AKT2	DQ198085	CAGAGAGCCGAGTCCTACAGAATAC	GTCATGGGTCTGGAAGGCATA

2.3.5.3 Polymerase Chain Reaction (Real Time PCR)

A mastermix without the sample cDNA was prepared as shown in Table 2.2. The Roche Lightcycler® 480 SYBR was used to amplify cDNA samples. For the standard curve, a pool of cDNA was made from all samples to be analysed. The standard curve was used to determine mRNA expression from Cp values. Expression was normalised to β-actin. All samples were analysed in triplicate, and had a non-reverse transcriptase control. A non-template control was included, which contained water and mastermix only. The mastermix was vortexed and centrifuged. 10 µl of mastermix was then added to 5 µl cDNA sample in a 384 well plate format (Roche,UK). A different mastermix was required for each gene of interest. 384 well plates were then sealed, spun down and run on a standard probe run protocol on the Lightcycler® 480 instrument (Roche Diagnostics Ltd, UK).

Table 2. 2 Reagents and cDNA added to the 384 well reaction plate

Reagent	Volume (µl)
Lightcycler® 480 SYBR	7.5
Forward primer (10µM)	0.45
Reverse primer (10µM)	0.45
cDNA template	5.0
ddH2O (Sigma, UK)	1.6
Total	15

2.4 Statistical Analysis

All data was analyzed using the Statistical Package for Social Sciences (version 18; SPSS, Inc., Chicago, IL, USA) and presented as mean ± SEM. The effects of pre-gestational and gestational dietary treatment on maternal data were assessed using a general linear model ANOVA with fixed factors of maternal diet, age and gestation day. Fetal data was analyzed by using a mixed model ANOVA with fixed factors of maternal diet and sex, and with random factor litter size. $P<0.05$ was considered as significant unless otherwise indicated. Only significant effects or interactions are included. Any not mentioned were therefore not significant.

3.0 THE EFFECTS OF CAFETERIA DIET ON MATERNAL ADAPTATION TO PREGNANCY AND FETAL DEVELOPMENT

3.1 Introduction

Obesity and related disorders have been identified as the most important health problems of this century *(Heitmann et al., 2009).* Prevalence of obesity and over-weight are increasing dramatically in Western societies and in developing nations that are transitioning to industrialized status *(World Health Organization, 2000).* Besides the strong relationship with the morbidities of diabetes, hypertension and cardiovascular disease, obesity represents an important economic disturbance to the health care system *(Oken, 2009).* The rate is higher in women than men and of even more concern is the dramatic rise in childhood overweight and obesity (*Lang et al., 2010).* It is clear that this situation requires more consideration for women of child-bearing age, as by 2050 it is estimated that 30 % of 21 to 30 year old and 47 % of 31 to 40 year old women will be obese *(Foresight Report, 2007).*

There is a large body of evidence showing that maternal obesity may impair the normal growth and development of the fetus and, more importantly, may result in adverse pregnancy outcomes such as stillbirths, neonatal deaths and congenital abnormalities *(Cedergen, 2004, Naeye, 1990, Stott-Miller etal., 2010 and Waller et al., 1994).* On the other hand, the long-term effects of maternal obesity on the health of the fetus are less clear. It

has been suggested that exposure to over-nutrition during neonatal life could be a predisposing factor for obesity during adult life *(Parsons et al., 2001 and Whitaker, 2001).* However, it is very difficult to elucidate the underlying mechanisms of this, as obesity is a multi-factorial disease and the interactions between intergenerational features and environmental factors may be crucial in the development of the obesity.

The intrauterine environment has important effects on the developing fetus. Exposure to suboptimal neonatal conditions has been linked with chronic diseases during adult life *(Painter et al., 2005).* However, early studies exploring this concept mainly focused on undernutrition and growth restriction during pregnancy. There is now growing evidence from animal studies showing that overnutrition and consumption of high amount of Western-style diets during pregnancy can result in metabolic syndrome in the offspring *(Khan et al., 2003 and Taylor and Poston, 2007).* Recently, Bayol *et al* have demonstrated that offspring exposed to maternal high fat, sugar, sodium and processed foods during their pre-natal and lactation life developed hyperphagia and greater weight gain by their adulthood *(Bayol et al., 2007).* Similarly, greater adiposity, disturbed glucose and lipid metabolism were shown in mice following feeding of highly energetic and fatty diets before and during pregnancy *(Samuelsson et al., 2008, Siemelink et al., 2002).*

In these studies one of the main questions that arose is whether the observed outcomes were due to maternal adiposity or the experimental diet itself, as the maternal feeding protocols started at confirmation of pregnancy

or the dams continued on the same diets during pre-mating and pregnancy. Recently, it has been reported that maternal adiposity, and not dietary fat *per se* is necessary for the programming effect of a high-fat diet on offspring. Among three study groups (high fat, low fat or high fat but pair fed to the caloric intake of low fat), only offspring of high fat dams exhibited greater adiposity, hyperleptinemia and insulin resistance *(White et al., 2009).* Another study used an overfeeding based model in rats utilizing intragastric feeding of diets via total enteral nutrition, transferring obese animals to a control diet at mating and cross fostering offspring to lean dams at birth *(Shankar et al., 2008).* According to the results, offspring of obese dams exhibited increased adiposity and insulin resistance in comparison to those from lean dams. This indicated an independent effect of maternal obesity.

3.2 Objectives

The study described in this chapter aimed to:

i. Develop a robust model for the evaluation of the independent effects of cafeteria diet induced obesity during pre-pregnancy and/or cafeteria diet during pregnancy.
ii. Assess the impact of cafeteria diet on maternal nutritional status, body composition and fecundity.
iii. Determine the effect of cafeteria diet on maternal adaptation to pregnancy and metabolic biomarkers
iv. Evaluate the effect of cafeteria diet on fetal and placental growth.

Data described in chapter has been published *(Akyol et al., 2009).*

3.3 Materials and Methods

This chapter describes data obtained from three different pre-natal trials focusing on the nutritional status of the pregnant rat and physiological adaptation to pregnancy.

3.3.1 Animal Procedures

3.3.1.1 Pilot trial

Tissues were collected at day 5 of pregnancy in animals exposed to chow or cafeteria diet since 6 weeks before mating. Detailed animal procedures for this trial were described at section 2.1.2.

3.3.1.2 Maternal trial

Tissues were collected at day 5 and 20 of pregnancy. Animals exposed to chow or cafeteria diet since 8 weeks before mating and half of the animals in each group were kept on the same diets while the rest of them switched to the alternative diet after mating. Plasma volume determination was performed in this trial. Fetal and placental weights were assessed from the pregnancies terminated on day 20. Detailed animal procedures for this trial were described at section 2.1.3.

3.3.1.3 Maternal data obtained from offspring trial

This section includes maternal data obtained from offspring trial. Detailed animal procedures for this trial were set out at section 2.1.4.

3.3.2 Biochemical Endpoints

Plasma non-fasted glucose, cholesterol and triglyceride concentrations were measured through an adaptation of glucose oxidase method and commercially available kits (as described in 2.2.3.1, 2.2.3.2 and 2.2.3.3). Soxhlet and Kjheldal methods were used to measure body fat and body nitrogen content, respectively (as described in 2.2.6).

3.3.3 Statistical Analysis

Data was analyzed using the Statistical Package for Social Sciences (version 18; SPSS, Inc., Chicago, IL, USA). Values are presented as mean ± SEM. For the pilot trial, a general linear model ANOVA was used with fixed factors of maternal diet and week of study. Maternal trial data was assessed using a general linear model ANOVA with fixed factors of pre-gestational diet, gestational diet, gestational time and week of study where applicable. It has been suggested that counting each offspring as a separate observation may lead to type I errors in experiments involving neonates with different litter sizes *(Festing, 2006).* Therefore, fetal data was analyzed by using a mixed model ANOVA with fixed factorsof maternal diet, gender and with litter size as a random factor. Where longitudinal data were available (for example, weekly body weights or energy intake), the week of study was used in a repeated-measures analysis. $P<0.05$ was considered as significant unless otherwise indicated. No post hoc tests were necessary. Differences between the groups shown in tables and figures represent main effects.

3.4 Results

3.4.1 Results of Pilot Study

All of the animals mated normally and pregnancy was confirmed by the observation of semen plug on the cage floor.

3.4.1.1 Nutrient Intakes

Figure 3.1 shows the energy intake of the animals throughout the study. The average energy intake of the cafeteria diet group (O) (282.29±6.89 Kj/day and 2.20±0.04 Kj/day/kg) was significantly higher than the control group (C) (252.21±6.89 Kj/day and 1.83±0.04 kj/day/kg) ($P<0.001$). This effect was most pronounced during the first and the second weeks of the study ($P<0.001$). Throughout the study, protein and carbohydrate intakes of cafeteria fed rats were significantly lower than in the control group (Protein: 2.80±0.07 g/day for C and 2.24±0.07 g/day for O, $P<0.001$ and carbohydrate: 11.67±0.32 g/day for C and 7.47±0.32 for O, $P<0.001$). As expected, the fat intake of the cafeteria diet group was dramatically higher (0.45±0.59 g/day for C and 3.31±0.59 g/day for O, $P<0.001$). Figure 3.2 shows average macronutrient intakes as a percentage of energy.

The amount of the energy intake from chow diet was significantly lower than cafeteria foods in cafeteria diet fed animals (23.52 ± 2.35 % from chow diet and 76.48 ± 2.35 from cafeteria diet, $P<0.05$) (Figure 3.3).

Figure 3. 1 Average energy intakes of control and cafeteria diet groups

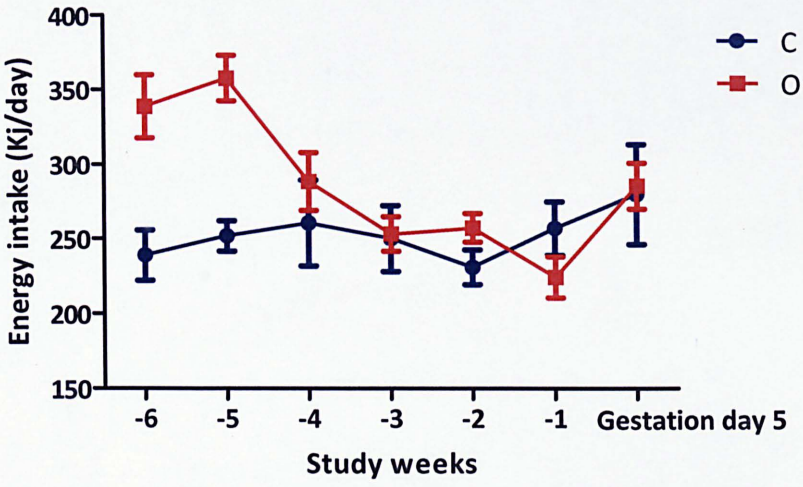


Figure 3.1 Average energy intakes of control and cafeteria diet groups. Data is shown as mean±SEM for n=4 per group. C: Chow diet and O: Cafeteria diet. There was a significant effect of maternal cafeteria diet (P<0.001). Duration of study had a significant influence on energy intake (P<0.001). There was a significant interaction between dietary treatment and study weeks.

Figure 3. 2 Average macro-nutrient intakes of control and cafeteria diet groups

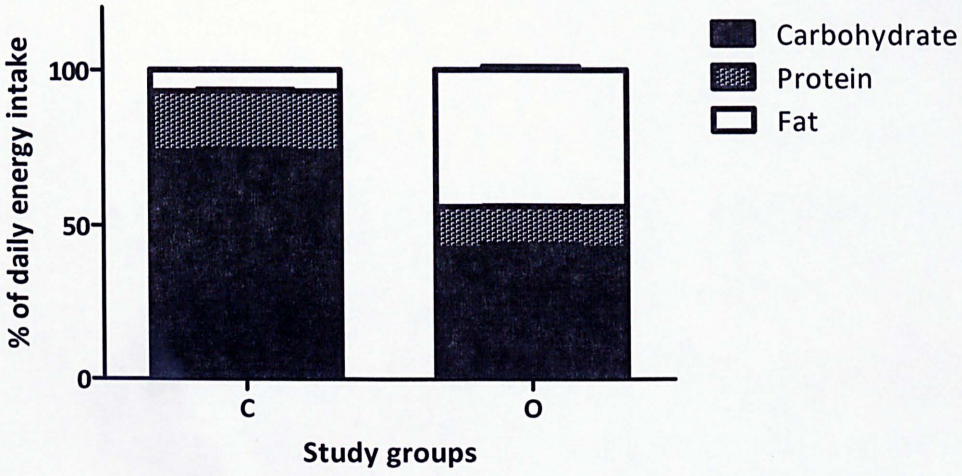


Figure 3.2 Average macro-nutrient intakes of control and cafeteria diet groups. Data is shown as mean±SEM for n=4 per group. C: Chow diet and O: Cafeteria diet. Dietary fat intake was significantly increased (P<0.001) and protein and carbohydrate intakes were significantly decreased (P<0.001) in cafeteria diet fed animals.

Figure 3. 3 Average percentage of energy from chow diet in study groups

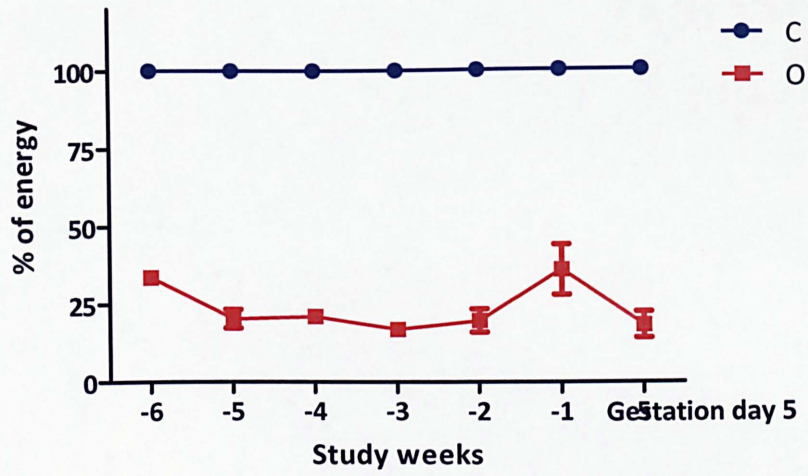


Figure 3.3 Average percentage of energy from chow diet in study groups. Data is shown as mean±SEM for n=4 per group. Percentage of energy coming from chow diet was significantly lower in cafeteria diet fed rats (P<0.05).

3.4.1.2 Body weight and body composition

All of the animals gained weight throughout the study. Despite the higher energy intake of the cafeteria diet group, body weight was not significantly different between the groups at day 5 of gestation (P>0.05) (Figure 3.4). However, cafeteria diet feeding significantly increased the mass of intrascapular brown adipose tissue, gonadal fat and peri-renal fat (P<0.001) (Figure 3.5). Intrascapular white adipose tissue weight was not significantly different between groups (P>0.05) (Figure 3.5). Whole body carcass analysis data showed that the cafeteria diet group had significantly higher levels of body fat (P<0.05) and significantly less body nitrogen and water than controls (P>0.05) (Figure 3.6).

Figure 3. 4 Average body weights of control and cafeteria diet groups

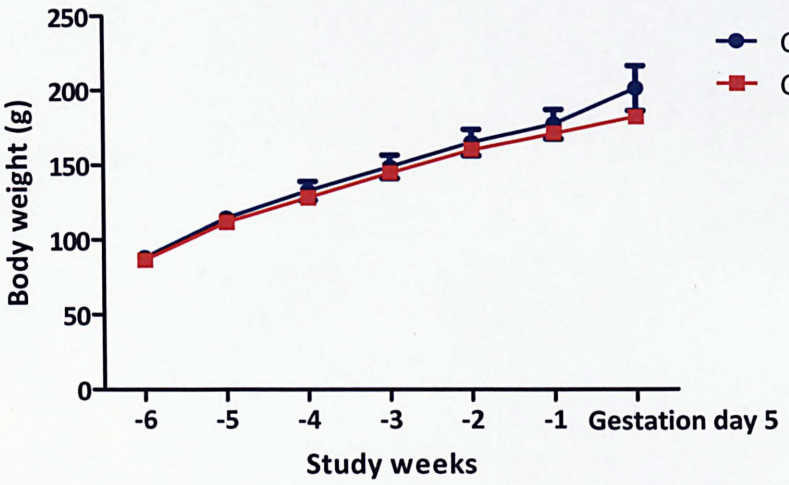


Figure 3.4 Average body weights of control and cafeteria diet groups. Data is shown as mean±SEM for n=4 per group. C: Chow diet and O: Cafeteria diet. Body weights of the animals did not differ significantly at the end of the study. Body weight significantly increased over time (P<0.05).

Figure 3. 5 Adipose tissue depost size in control and cafeteria diet groups

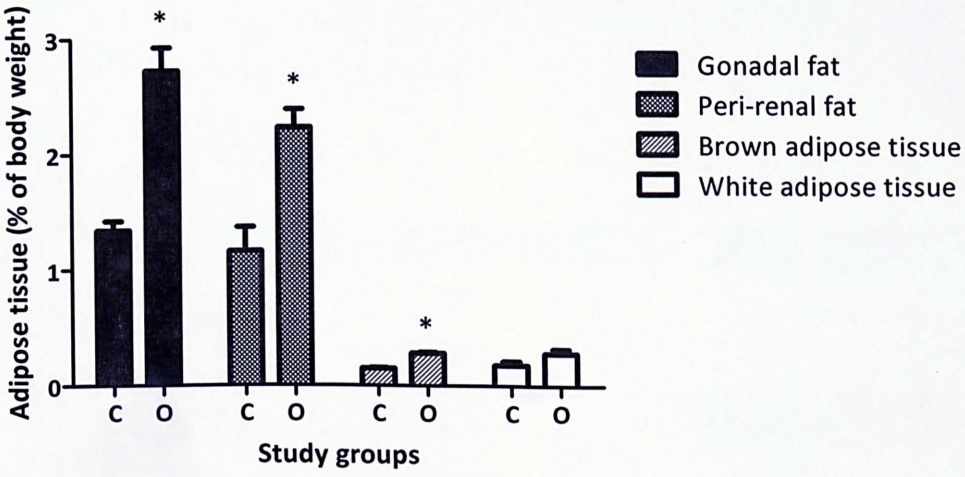


Figure 3.5 Adipose tissue depost size in control and cafeteria diet groups. Data is shown as mean±SEM for n=4 per group. C: Chow diet and O: Cafeteria diet. * indicates the significant effect of cafeteria diet on gonadal fat, peri-renal fat and intrascapular brown adipose tissue by day 5 of pregnancy (P<0.001).

Figure 3. 6 Whole body carcass analysis of control and cafeteria diet groups

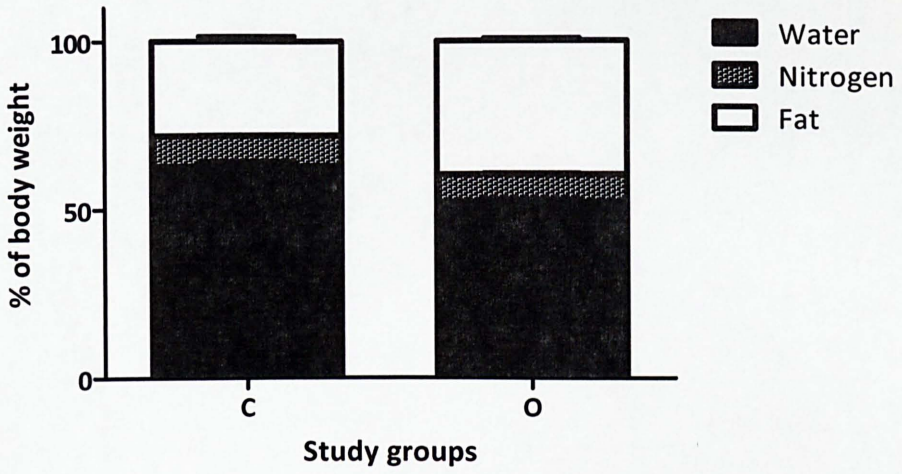


Figure 3.6 Whole body carcass analysis of control and cafeteria diet groups. Data is shown as mean±SEM for n=4 per group. C: Chow diet and O: Cafeteria diet. Total body fat was significantly increased ($P<0.05$) and body nitrogen and water were significantly decreased ($P<0.05$) in cafeteria diet fed animals at day 5 of gestation.

3.4.1.3 Plasma Volume Measurement

No significant effect of the maternal diet was found on plasma volume at day 5 of gestation ($P>0.05$) (Figure 3.7).

Figure 3. 7 Plasma volumes of control and cafeteria diet groups on day 5 of pregnancy

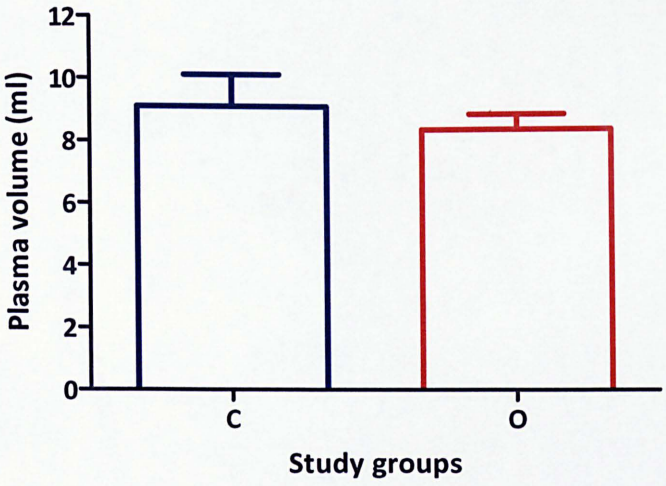


Figure 3.7 Plasma volumes of control and cafeteria diet groups on day 5 of pregnancy. Data is shown as mean±SEM for n=4 per group. C: Chow diet and O: Cafeteria diet. Plasma volume was not significantly different between the groups on day 5 of gestation (P>0.05).

3.4.2 Results of the Maternal Trial

3.4.2.1 Nutrient Intakes

Eight weeks of cafeteria diet feeding before mating resulted in significantly higher overall energy intake (P<0.001) (Figure 3.8 and Table 3.1). The animals which were fed cafeteria diet during the pre-gestational period consumed significantly lower amounts of chow diet (P<0.001) (Figure 3.8). When the nutrient composition of the foods consumed was analysed, the fat intake of the cafeteria fed animals was dramatically higher whereas protein and carbohydrate intakes were lower (P<0.001) (Table 3.1). The sodium

intake was 2.7 times greater in cafeteria diet group than in the control group ($P<0.001$) (Table 3.1). Energy and nutrient intake data were also calculated per kg of body weight to assess whether the differences observed in nutrient intake data were due to rats having greater body mass. These effects remained significant when the nutrient intake was expressed per kg body weight of nutrient intake ($P<0.001$) (Table 3.1).

During pregnancy, the cafeteria diet influenced energy and nutrient intakes of the animals irrespective of their pre-gestational diet. However, pre-gestational cafeteria diet significantly influenced the amount of chow diet consumed by cafeteria diet groups in pregnancy. The group OO consumed significantly lower amounts of chow diet than the group CO ($P<0.001$) (Figure 3.8). Energy, fat and sodium intakes of the animals which had the cafeteria diet during gestational period (CO and OO) were significantly higher and protein and carbohydrate intakes were significantly lower than in the animals which had chow diet during gestational period (CC and OC) ($P<0.05$) (Figure 3.9 and Table 3.1). These effects remained significant when the data was expressed per kg body weight of nutrient intake ($P<0.05$) (Table 3.1). Pre-gestational cafeteria diet feeding influenced the energy and nutrient intakes during gestational period, but only when expressed as per kg body weight. Animals which had been fed the cafeteria diet during pre-gestational period (OC and OO) exhibited significantly lower intakes of energy, protein and carbohydrates per kg body weight during pregnancy when compared to the corresponding groups of animals (CC and OC) which had been fed chow diet

during pre-gestational period (CC and CO) ($P<0.05$) (Table 3.1). Fat intake was not found to be significantly different in this context.

The effects of cafeteria diet feeding on macro-nutrient composition during pre-pregnancy and pregnancy periods are also shown in Figure 3.9. Cafeteria diet animals consumed significantly a higher percentage of their daily energy intake as fat, and significantly less as protein and carbohydrate ($P<0.001$).

Table 3. 1 Average maternal nutrient intakes during pre-gestational and gestational periods

	Pre-gestational intakes				Gestational intakes							
	C (n=23)		O (n=23)		CC (n=12)		CO (n=11)		OC (n=11)		OO (n=12)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Energy intake												
(KJ/day/kg)	1870.0	49.57	1970.8*	49.57	1364.1	37.39	1479.1†	38.19	1199.0‡	39.96	1340.4†‡	37.38
(KJ/day)	250.61	2.34	281.71*	2.34	330.84	11.47	379.06†	11.72	311.15	12.27	379.96†	11.47
Fat intake												
(g/day/kg)	3.43	0.05	21.87*	0.51	2.44	0.12	16.93†	0.54	2.07	0.12	16.28†	0.80
(g/day)	0.45	0.001	3.09*	0.07	0.58	0.03	4.23†	0.17	0.53	0.03	4.45†	0.21
Protein intake												
(g/day/kg)	21.66	0.29	16.96*	0.25	16.17	0.80	11.16†	0.64	13.29‡	0.83	9.54†‡	0.23
(g/day)	2.78	0.05	2.22*	0.05	3.81	0.19	2.81†	0.20	3.41	0.23	2.63†	0.13
CHO intake												
(g/day/kg)	85.49	1.21	56.61*	1.18	59.06	2.64	37.98†	2.45	48.87‡	2.99	33.49†‡	1.40
(g/day)	11.1	0.22	7.56*	0.22	14.0	0.63	9.55†	0.67	12.58	0.85	9.16†	0.41
Na intake												
(g/day/kg)	0.176	0.040	0.454*	0.0011	0.134	0.0019	0.338†	0.0011	0.109‡	0.007	0.285†‡	0.001
(g/day)	0.023	0.001	0.062*	0.004	0.031	0.004	0.084†	0.003	0.028	0.002	0.079†	0.004

Table 3.1 Average maternal nutrient intakes during pre-gestational and gestational periods. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet, CHO: Total carbohydrate, Na: Sodium. * indicates the significant effect of cafeteria diet on nutrient intakes during pre-gestational period in comparison to controls ($P<0.001$). † indicates the significant effect of cafeteria diet on nutrient intakes during gestational period in comparison to controls during gestational period ($P<0.05$). ‡ indicates the significant effect of pre-gestational cafeteria diet on nutrient intakes during gestational period in comparison to controls which had chow diet during pre-gestational period ($P<0.05$).

Figure 3. 8 Average maternal energy intake and percentage of energy from chow diet throughout the study

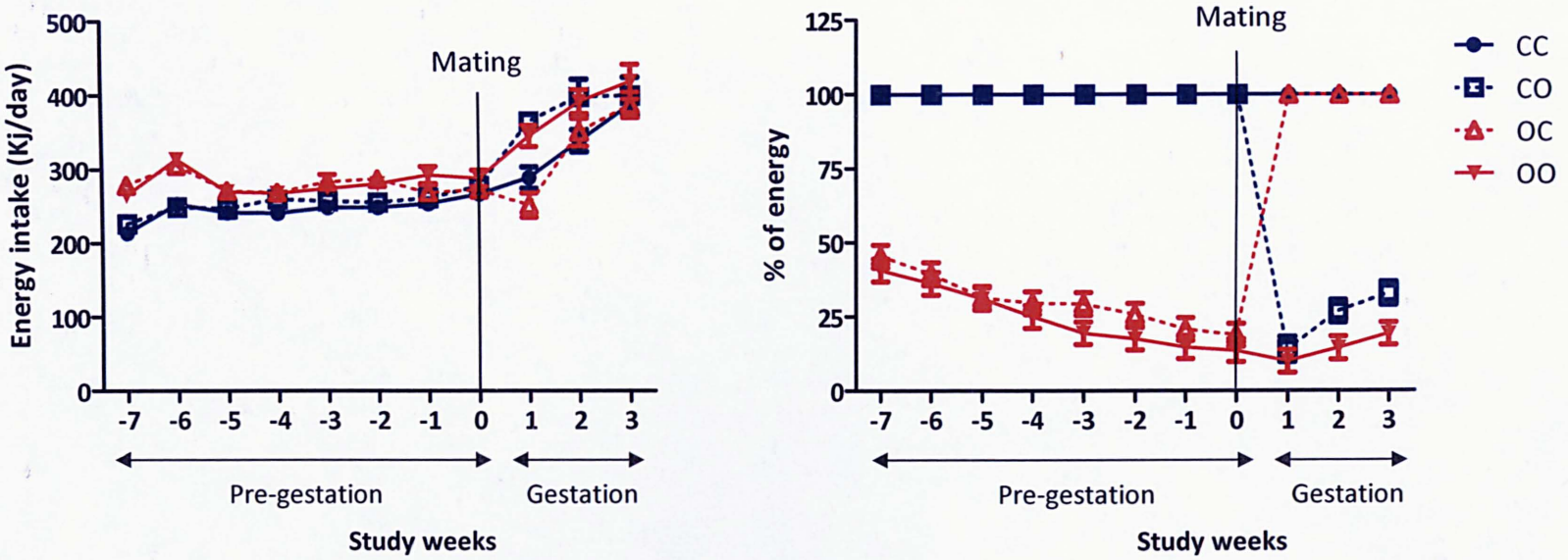


Figure 3.8 Average maternal energy intake and percentage of energy from chow diet throughout study. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6-12), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6-12), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6-11), OO: Cafeteria diet during pre-gestation and gestation (n=6-12). Study weeks between -7 and 0 represent the pre-gestational treatment. Conception is at 0. Study weeks between 1 and 3 represent pregnancy. Pre-gestational and gestational cafeteria diet intake significantly affected total energy intake ($P<0.05$). During pregnancy the group OO ate significantly less chow diet than the group CO ($P<0.001$).

Figure 3. 9 Average maternal nutrient intakes as percentage of daily energy intake

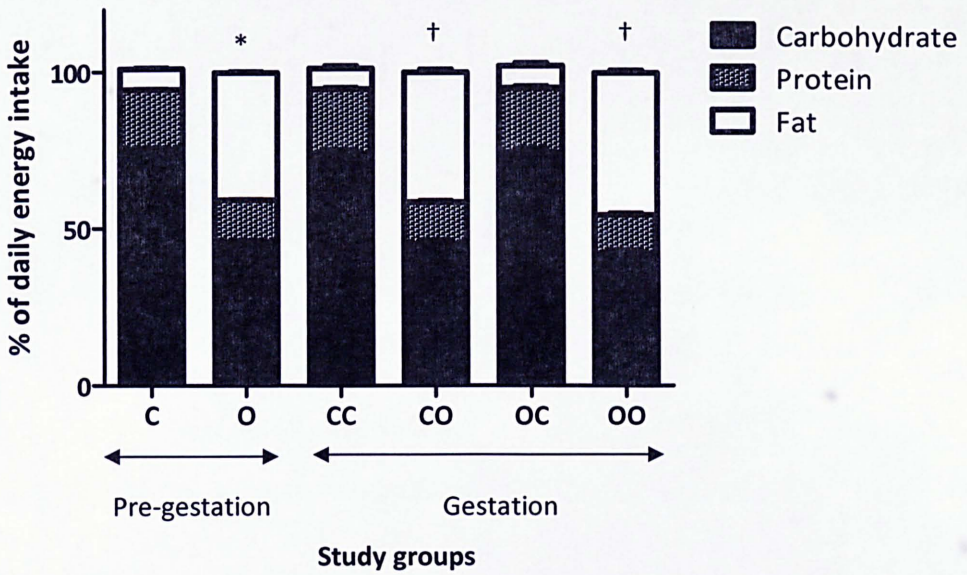


Figure 3.9 Average maternal nutrient intakes as percentage of daily enegy intake. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6-12), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6-12), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6-11), OO: Cafeteria diet during pre-gestation and gestation (n=6-12). Pre-gestational (* $P<0.001$) and gestational († $P<0.001$) cafeteria diet significantly increased fat and significantly lowered carbohydrate and protein intakes when the data was expressed as percentage of daily energy intake.

3.4.2.2 Maternal body weight and body composition

All of the animals gained weight throughout the study. At the end of the pre-gestational period the animals which were fed the cafeteria diet (OC and OO) were significantly heavier than the control animals (CC and CO) ($P<0.05$) (Figure 3.10). The amount of the weight gained during this pre-mating period was significantly more in the cafeteria diet animals (116±24 g

for control and 142±30 g for cafeteria diet) (P<0.001). Furthermore, weight gain during pregnancy was also higher in these animals (90±27 g for control and 115±33 g for cafeteria diet (P<0.001). Consequently, the animals which had the cafeteria diet both for pre-gestational and gestational period (OO) had the highest body weight at the end of the study (Figure 3.10). The lowest gestational weight gain was observed in the animals which had the cafeteria diet during pre-gestational period and switched to chow diet during gestation (OC).

Figure 3. 10 Average maternal body weights throughout the study

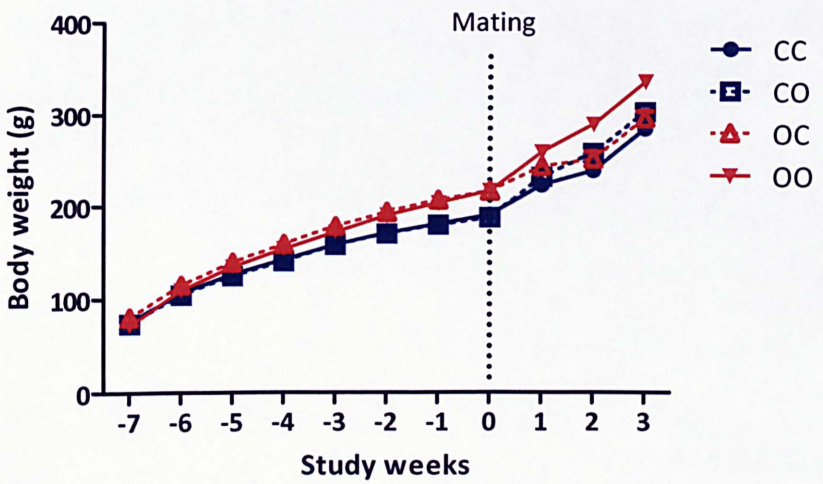


Figure 3.10 Average maternal body weights throughout study. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5), OO: Cafeteria diet during pre-gestation and gestation (n=6). Study weeks between -7 and 0 represent the pre-gestational treatment. Conception is at 0. Study weeks between 1 and 3 represent pregnancy. Animals fed cafeteria diet during pre-gestational or gestational period had significantly greater weight gain (P<0.001).

Pre-gestational cafeteria diet feeding significantly increased the size of maternal adipose tissue depots by day 5 and day 20 of gestation, irrespective of pregnancy diets ($P<0.05$) (Table 3.2). Although there was less gonadal fat and intrascapular white adipose tissue in the animals which switched to chow diet during gestation (OC), these rats still had significantly larger fat depots at day 20 of gestation when compared to the CC group. Similarly, initiating cafeteria diet feeding during pregnancy resulted in heavier fat depots but this effect did not reach significance for gonadal fat (Table 3.2). The animals fed chow diet during pre-gestation and cafeteria diet during gestation (CO) had larger fat depots at both day 5 and 20 ($P<0.05$). Gestational age was only found to significantly influence peri-renal fat which was increasingly deposited in later pregnancy (Table 3.2).

The increase in the size of fat depots in the animals fed cafeteria diet was also reflected in the total body fatness. Cafeteria diet during the pre-gestational period significantly increased the total body fatness, whereas body nitrogen and water levels were significantly decreased ($P<0.05$) (Figure 3.11). Gestational cafeteria diet was also effective in inducing a higher amount of body fat and lower body nitrogen and water levels in comparison to control animals during gestational period ($P<0.05$) (Figure 3.11). The total body fat of the animals which had chow diet during pre- gestational period and then switched to cafeteria diet during gestation (CO) continued to increase from day 5 (38 % of body fatness) to day 20 (46 % of body fatness). In contrast, total body fat of the animals which had cafeteria diet during pre-

gestational period and then switched to chow diet during gestation (OC) remained similar from day 5 (41 %) to day 20 (42 %). Despite this their total body fatness remained significantly higher than in the controls ($P<0.05$). Thus, animals which had the cafeteria diet both during pre-gestational and gestational periods (OO) had the highest body fat content both at day 5 and 20 (Figure 3.11).

Table 3. 2 Maternal adipose tissue mass on day 5 and 20 of pregnancy

	Fat depot as percentage of body weight (%)							
	Gonadal fat		Peri-renal fat ‡		BAT		WAT	
Maternal diet and gestational age	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CC, day 5	1.84	0.18	1.33	0.13	0.17	0.03	0.19	0.04
CO, day 5	2.00	0.16	1.83†	0.17	0.25†	0.03	0.26†	0.05
OC, day 5	3.23*	0.5	2.19*	0.31	0.24*	0.03	0.30*	0.04
OO, day 5	3.20*	0.54	2.98*†	0.58	0.32*†	0.03	0.35*†	0.04
CC, day 20	2.24	0.32	2.06	0.17	0.15	0.03	0.22	0.04
CO, day 20	2.52	0.46	2.51†	0.28	0.21†	0.03	0.32†	0.04
OC, day 20	2.49*	0.57	2.32*	0.31	0.30*	0.03	0.25*	0.05
OO, day 20	3.36*	0.44	3.72*†	0.42	0.27*†	0.03	0.39*†	0.04

Table 3.2 Maternal adipose tissue mass on day 5 and 20 of pregnancy. Data is shown as mean±SEM. CC day 5 or day 20: Chow diet during pre-gestation and gestation (n=6), CO day 5 or day 20: Chow diet during pre-gestation and cafeteria diet during gestation (n=6), OC day 5 or day 20: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 or 6), OO day 5 or day 20: Cafeteria diet during pre-gestation and gestation (n=6), BAT: Intrascapular brown adipose tissue, WAT: Intrascapular white adipose tissue. * indicates the significant difference of cafeteria diet fed animals during pre-gestational period in comparison to controls of pre-gestational period ($P<0.05$). † indicates the significant difference of cafeteria diet fed animals during gestational period in comparison to controls during gestational period ($P<0.05$). ‡ indicates the significant effect of gestational age (day 5 or 20) on peri-renal fat ($P<0.05$). Pre-gestational cafeteria diet significantly increased the size of fat pads both on day 5 and 20 of the gestation ($P<0.05$). Gestational cafeteria diet also significantly increased the size of fat pads both on day 5 and 20 of pregnancy except gonadal fat depot ($P<0.05$). Gestational age significantly influenced peri-renal fat depot ($P<0.05$).

Figure 3. 11 Whole body carcass analyses on day 5 and 20 of pregnancy

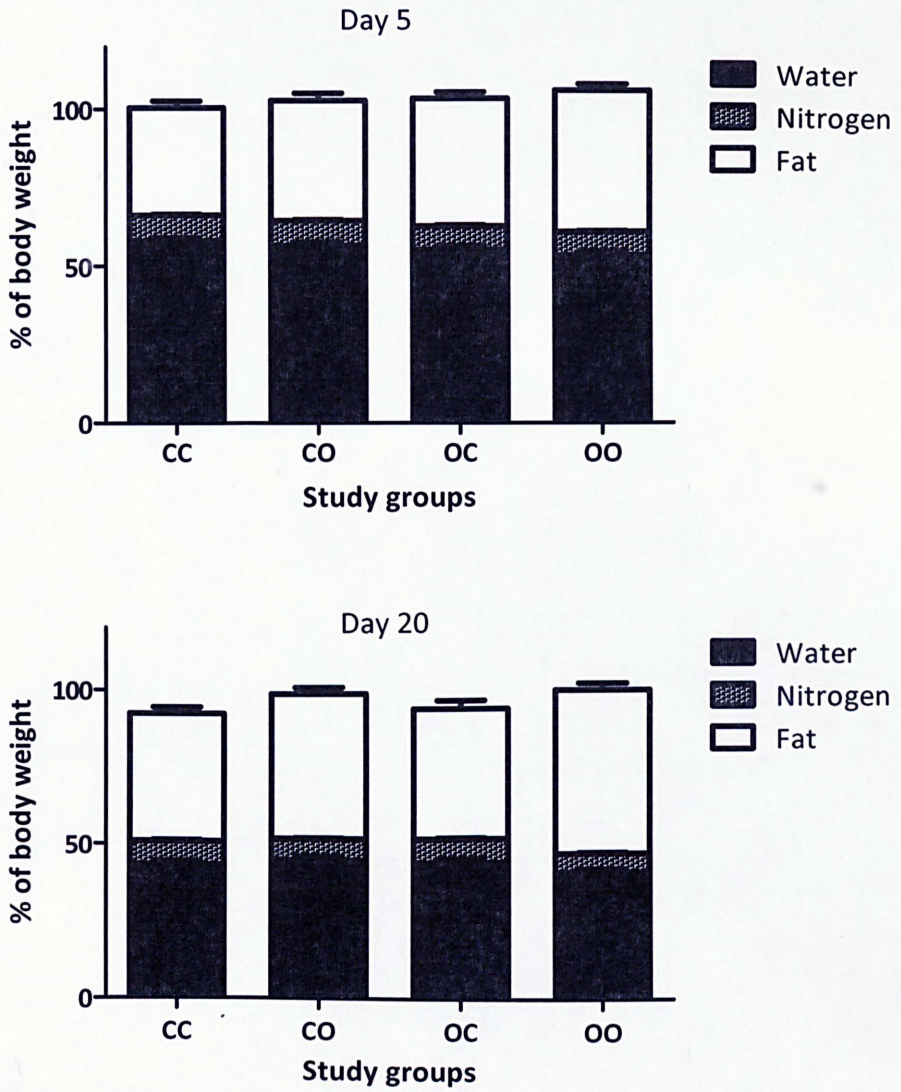


Figure 3.11 Whole body carcass analyses on day 5 and 20 of gestation. Data is shown as mean±SEM. Data was analysed altogether including the effect of pregnancy day. CC: Chow diet during pre-gestation and gestation (n=6), CO: Chow diet during pre-gestation and cafeteria diet during. Cafeteria diet during pre-gestation and chow diet during gestation (n=5), OO: Cafeteria diet during pre-gestation and gestation (n=6). Pre-gestational cafeteria diet was found to significantly influence total body fat, and the body nitrogen and water both on day 5 and 20 of gestation ($P<0.05$). Gestational cafeteria diet feeding also influenced body composition leading to an increase in total body fat and decrease in body nitrogen and water ($P<0.05$). This effect became stronger by day 20 of gestation ($P<0.05$).

3.4.2.3 Plasma metabolites

Despite the fact that the animals fed cafeteria diet during the pre-gestational and/or gestational period were relatively obese (based on adipose depot size), plasma metabolites did not exhibit a marked disturbance. Pre-gestational and gestational dietary treatments did not elicit any separate effects on plasma glucose, cholesterol and triglyceride concentrations among the study groups. The gestational age of the mothers (Day 5 or 20) was found to significantly influence plasma cholesterol and triglyceride concentrations which were increased by day 20 of gestation when compared to day 5 ($P<0.05$) (Table 3.3). There was a tendency towards an interaction between the pre-gestational and gestational cafeteria diet, which was indicating the groups CO, OC and OO had higher plasma glucose concentrations compared to control group CC on day 20 of pregnancy ($P<0.06$) (Table 3.3).

3.4.2.4 Reproductive Outcome

All of the animals mated normally and only one of them failed to carry their pregnancy to the end of the study. Litter size was not affected by pre-gestational and/or gestational dietary treatment (CC: 12.3±0.5, CO: 11.7±0.5, OC: 13.0±0.45, OO: 11.3±1.3). Plasma volume measurement was performed to assess whether the physiological adaptation to pregnancy may be affected by obesity or high-fat diet. The data showed that there was no significant difference between the groups (Figure 3.12). Gestational age was found to influence plasma volume levels, as all of the groups exhibited an increase between day 5 and day 20 of gestation ($P<0.001$).

Table 3. 3 Maternal plasma metabolites on day 5 and 20 of pregnancy

	Glucose (mmol/L)				Cholesterol (mmol/L)				Triglycerides (mmol/L)			
	Day 5		Day 20		Day 5		Day 20		Day 5		Day 20	
Maternal diet	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CC	3.49	0.43	2.09	0.13	1.54	0.11	2.21*	0.23	0.85	0.18	2.05*	0.49
CO	2.75	0.23	3.11	0.22	1.11	0.21	1.77*	0.19	0.43	0.19	1.48*	0.46
OC	3.54	0.27	2.96	0.42	1.55	0.11	1.88*	0.30	0.65	0.15	2.99*	0.5
OO	3.72	0.85	2.91	0.26	1.60	0.10	2.08*	0.14	0.52	0.16	2.20*	0.63

Table 3.3 Maternal plasma metabolites on day 5 and 20 of pregnancy. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 or 6), OO: Cafeteria diet during pre-gestation and gestation (n=6). * indicates the significant effect of gestational age on cholesterol and triglycerides. Gestational age was found to significantly influence plasma cholesterol ($P<0.001$) and triglyceride concentrations ($P<0.05$) from day 5 to 20 of gestation. No individual effects of pre-gestational or gestational dietary treatment were found on plasma metabolites.

Figure 3. 12 Plasma volumes on day 5 and 20 of pregnancy

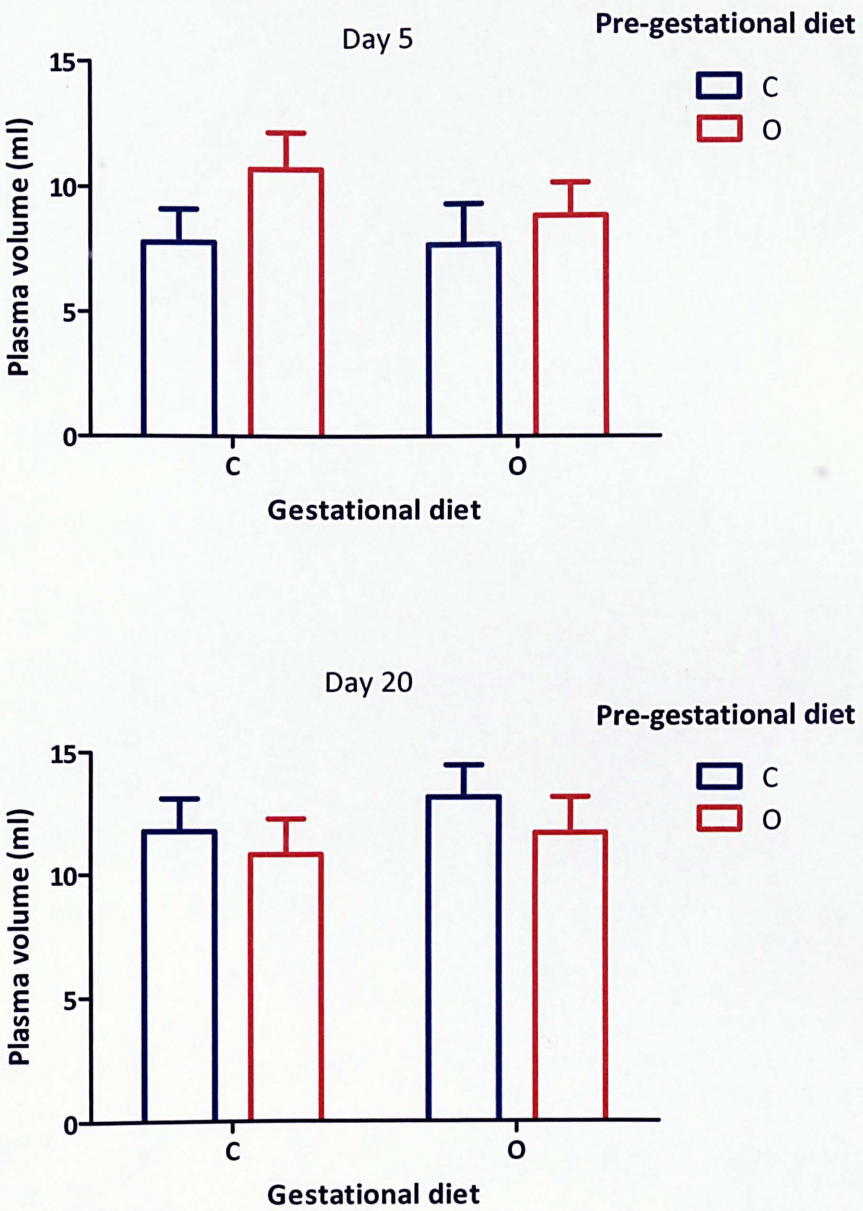


Figure 3.12 Plasma volumes on day 5 and 20 of pregnancy. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet. n=5 or 6 in each group. Plasma volume was significantly influenced by gestational age ($P<0.001$). There was no significant effect of pre-gestational or gestational dietary treatment.

3.4.2.5 Fetal and placental growth

Fetal and placental weights were analysed from the animals which were culled on day 20 of gestation. The marked effects of pre-gestational cafeteria diet on maternal body composition were reflected in the development of the fetuses. For instance, fetuses of the animals which had cafeteria diet during pre-gestational period (OC and OO) had significantly lower body weights than the offspring of control animals (CC and CO) ($P<0.05$) (Figure 3.13). Although cafeteria diet feeding in pregnancy did not show any significant effect on fetal growth, it significantly influenced placental development. Animals which were fed by cafeteria diet during gestational period (CO and OO) had significantly lower placental weights ($P<0.001$) (Figure 3.14). These alterations in the fetal body weight and placental weight were reflected by differences in the fetal:placental weight ratio. Pre-gestational cafeteria diet group significantly decreased and pregnancy cafeteria diet significantly increased this ratio ($P<0.05$) (Figure 3.15). The lowest fetal:placental ratio was therefore observed in the OC group, and highest in CO group. In addition to this, when the fetal organ proportions were evaluated, only left kidney and brain size were found to be affected by pre-gestational or gestational dietary treatment (Table 3.3). Gestational cafeteria diet feeding significantly reduced the left kidney mass ($P<0.05$) whereas an interaction between pre-gestational and gestational cafeteria diet feeding significantly increased the brain mass of the group OO ($P<0.001$) (Table 3.3).

Figure 3. 13 Average fetal body weights on day 20 of pregnancy

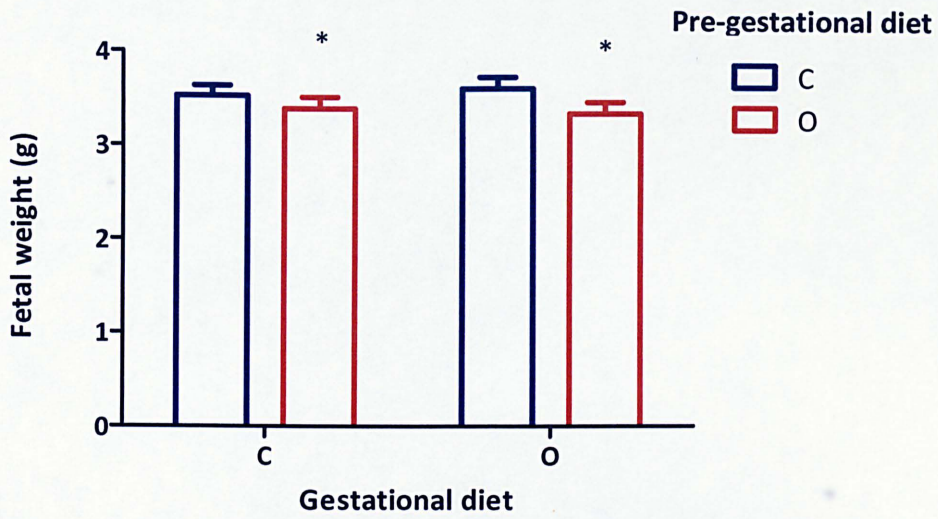


Figure 3.13 Average fetal body weights on day 20 of pregnancy. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet. n=59-73 in each group. * indicates the significant effect of pre-gestational cafeteria diet when compared to controls during pre-gestational period. Fetal weight was significantly lower in the maternal groups which had the cafeteria diet during pre-gestational period ($P<0.05$).

Figure 3. 14 Average placental weights on day 20 of pregnancy

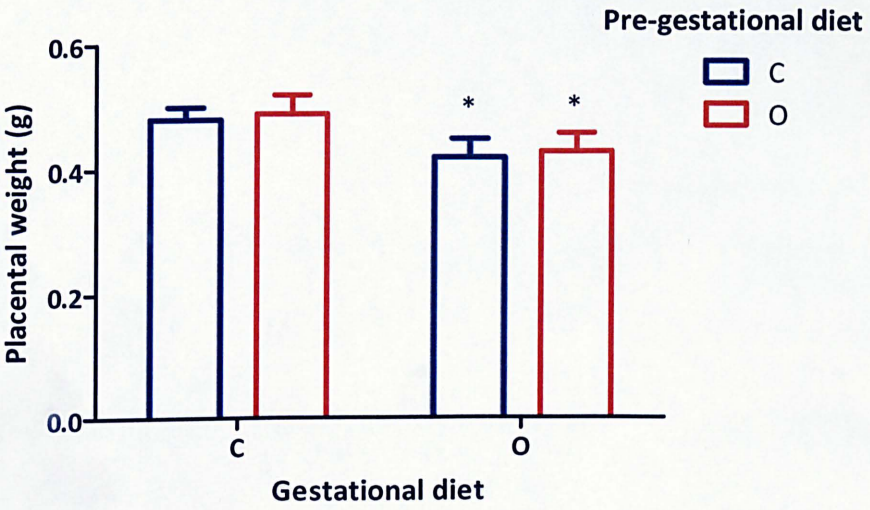


Figure 3.14 Average placental weights on day 20 of pregnancy. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet. n=59-73 in each group. * indicates the significant effect of pregnancy cafeteria diet when compared to controls during gestational period. The placental weight was significantly lower in the groups which had the cafeteria diet during pregnancy ($P<0.001$).

Figure 3. 15 Fetal/placental ratios on day 20 of pregnancy

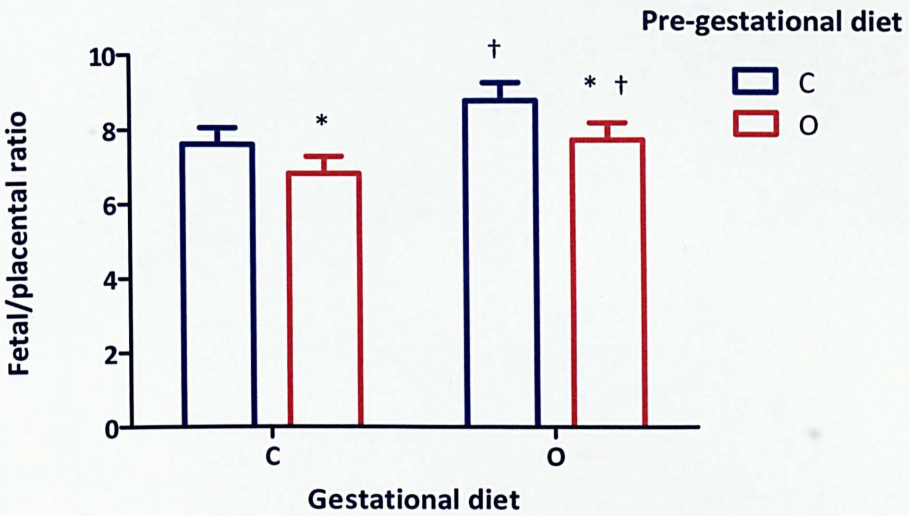


Figure 3.15 Fetal/placental ratios by day 20 of pregnancy. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet. n=59-73 in each group. * indicates the significant effect of pre-gestational cafeteria diet when compared to controls during pre-gestational period. † indicates the significant effect of gestational cafeteria diet when compared to controls during pregnancy. The fetal:placental ratio was significantly lower in the groups which had the cafeteria diet during pre-gestational period and significantly higher in the groups which had the cafeteria diet during pregnancy ($P<0.001$).

Table 3. 4 Fetal organ sizes with respect to fetal body weight on day 20 of pregnancy

	Organ weight/body weight (%)									
	Liver		Brain		Heart		Left kidney		Right kidney	
Maternal diet	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CC	7.21	0.13	3.70	0.09	0.52	0.02	0.50	0.04	0.38	0.02
CO	6.94	0.14	3.58	0.09	0.51	0.02	0.36†	0.04	0.38	0.02
OC	6.90	0.15	3.87	0.10	0.57	0.02	0.42	0.05	0.44	0.02
OO	6.82	0.15	4.24‡	0.10	0.52	0.02	0.36†	0.05	0.35	0.02

Table 3.4 Fetal organ sizes with respect to fetal body weight on day 20 of pregnancy. Data is shown as mean±SEM for n=59-73 per group. † indicates the significant effect of gestational cafeteria diet feeding when compared to control chow diet animals during gestation. ‡ indicates the significant interaction between pre-gestational and gestational diets. Gestational cafeteria diet feeding significantly reduced the left kidney mass ($P<0.05$). The interaction between pre-gestational and gestational diets significantly influenced brain development ($P<0.001$).

3.4.3 Maternal Data Obtained From Offspring Trial

3.4.3.1 Pre-gestational and gestational periods

In parallel with the previous experiment (Section 3.4.2), pre-gestational cafeteria diet for 8 weeks significantly increased the energy intake of the mothers ($P<0.001$) (Table 3.5). The percentage of energy coming from chow diet was significantly lower in cafeteria diet fed animals ($P<0.05$) (Figure 3.17). As a result, total fat and saturated fatty acid intakes of the cafeteria diet group was dramatically increased ($P<0.001$) (Table 3.5). Although total carbohydrate intake was lower in these animals, sugar intake remained significantly higher ($P<0.001$) (Table 3.5). Correspondingly, protein intake was significantly decreased ($P<0.001$) (Table 3.5). Sodium intake was 3 times greater in cafeteria diet animals ($P<0.001$) (Table 3.5). These effects remained significant when data were expressed as per kg of the body weights ($P<0.001$) (Table 3.5).

Similarly, during pregnancy, cafeteria diet feeding (CO and OO) increased energy, fat, saturated fatty acids, sugar and sodium intakes whereas total carbohydrate and protein intakes were lower than in chow fed animals ($P<0.001$) (Table 3.5). In our previous maternal experiment nutritional intakes during pregnancy were influenced by pre-gestational cafeteria diet only when the data was expressed as per kg of the body weights (Section 3.4.2.1). In this study, pre-gestational cafeteria diet affected energy, carbohydrate and protein intakes irrespective of the analysis approach and, similarly, all of the other dietary components were observed to be influenced

by pre-gestational cafeteria diet when the data was expressed per kg of the body weight during pregnancy ($P<0.001$) (Table 3.5). Animals fed cafeteria diet during pregnancy had significantly lower intakes of chow diet than control animals ($P<0.001$) (Figure 3.17). However, pre-gestational diet did not influence this proportion during pregnancy ($P>0.001$) (Figure 3.17).

Table 3. 5 Average maternal nutrient intakes during pre-gestational and gestational periods

	Pre-gestational intakes				Gestational intakes							
	C (n=29)		O (n=35)		CC (n=11)		CO (n=18)		OC (n=20)		OO (n=15)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Energy intake												
(KJ/day/kg)	1483.26	20.65	1961.08*	20.97	1104.88	38.04	1404.53†	35.62	896.12‡	35.62	1259.05†‡	39.38
(KJ/day)	214.88	2.79	288.74*	2.84	272.85	8.35	389.48†	8.09	256.31‡	8.09	355.27†‡	8.81
Fat intake												
(g/day/kg)	6.17	0.35	23.09*	0.35	4.67	0.45	17.29†	0.42	3.77‡	0.42	16.24†‡	0.46
(g/day)	0.89	0.04	3.52*	0.04	1.16	0.09	4.79†	0.09	1.08	0.09	4.59†	0.10
Protein intake												
(g/day/kg)	19.28	0.36	13.90*	0.37	14.71	0.44	11.68†	0.41	11.87‡	0.41	10.64†‡	0.46
(g/day)	2.79	0.03	2.24*	0.04	3.64	0.09	3.24†	0.09	3.40‡	0.09	2.99†‡	0.09
CHO intake												
(g/day/kg)	58.90	0.96	40.73*	0.98	44.62	1.39	33.78†	1.30	36.01‡	1.30	28.66†‡	1.44
(g/day)	8.53	0.13	6.38*	0.13	11.05	0.32	9.38†	0.31	10.31‡	0.31	8.08†‡	0.34
SFA intake												
(g/day/kg)	0.99	0.31	9.92*	0.32	0.75	0.17	6.36†	0.16	0.603	0.16	5.99†	0.18
(g/day)	0.14	0.03	1.44*	0.03	0.19	0.04	1.77†	0.04	0.17	0.04	1.70†	0.05

Table 3.5 Average maternal nutrient intakes during pre-gestational and gestational periods (continued)

	Pre-gestational intakes				Gestational intakes							
	C (n=29)		O (n=35)		CC (n=11)		CO (n=18)		OC (n=20)		OO (n=15)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Sugar intake												
(g/day/kg)	5.07	0.66	17.60*	0.67	3.84	0.49	12.32†	0.46	3.09‡	0.46	11.07†‡	0.50
(g/day)	0.73	0.07	2.71*	0.07	0.95	0.15	3.42†	0.15	0.89	0.15	3.15†	0.16
Na intake												
(g/day/kg)	0.24	0.05	0.72*	0.05	0.18	0.001	0.39†	0.001	0.14‡	0.001	0.36†‡	0.001
(g/day)	0.034	0.004	0.096*	0.004	0.045	0.002	0.106†	0.002	0.041	0.002	0.102†	0.002

Table 3.5 Average maternal nutrient intakes during pre-gestational and gestational periods. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet, CHO: Total carbohydrate, SFA: Saturated fatty acids, Na: Sodium. * indicates the significant effect of pre-gestational cafeteria diet on nutrient in comparison to pre-gestational controls ($P<0.001$). † indicates the significant effect of cafeteria diet on nutrient intakes during gestational period in comparison to controls during gestational period ($P<0.05$). ‡ indicates the significant effect of pre-gestational cafeteria diet on nutrient intakes during gestational period in comparison to controls which had chow diet during pre-gestational period ($P<0.05$).

All of the animals gained weight throughout the study. Consistent with our previous study, at the end of the pre-gestational period the animals which were fed cafeteria diet (OC and OO) had significantly greater body weights than the control groups (CC and CO) (Figure 3.16). The amount of body weight gained during these weeks was significantly different between the chow and cafeteria diet fed groups (126 ± 43 g for C and 168 ± 30 g for O) ($P<0.001$). Similarly, gestational weight gain was also significantly higher in the cafeteria diet groups (101±11 for CO and 93±61 for OO) than in controls (65±15 g for CC and 67±92 g for OC) ($P<0.001$). As a result, the animals which had cafeteria diet during both pre-gestational and gestational periods had the highest body weight (273 ± 9.50 g). In this study there was also a significant interaction between pre-gestational and gestational diets leading the groups CO and OC to be heavier than control CC group by the end of gestation ($P<0.05$) (Figure 3.16).

Figure 3. 16 Average maternal body weights during pre-gestational and pregnancy periods

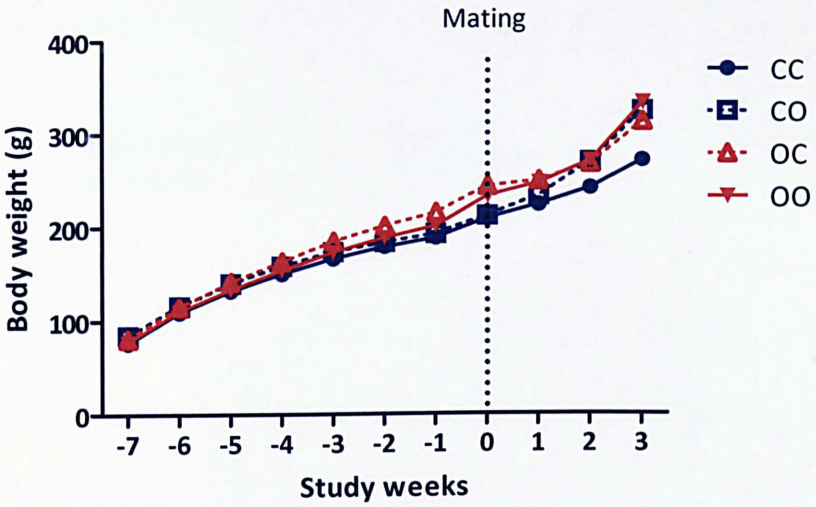


Figure 3.16 Average maternal body weights during pre-gestational and pregnancy gestation (n=11), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=18), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=20), OO: Cafeteria diet during pre-gestation and gestation (n=15). Study weeks between -7 and 0 represent the pre-gestational treatment. Conception is at 0. Study weeks between 1 and 3 represent pregnancy. Cafeteria diet fed animals during pre-gestational or gestational period had significantly higher levels of weight ($P<0.001$). There was a significant interaction between pre-gestational and gestational diets resulting in increased body weights for the groups CO and OC when compared to CC ($P<0.05$).

3.4.3.2 Chow Diet During Lactation

All of the animals increased their energy intake during lactation (Figure 3.17). When the dietary treatment was chow, energy intakes of the mothers which had been fed cafeteria diet during pregnancy (CO and OO) were found to be significantly decreased compared to the pregnancy control animals during lactation ($P<0.05$) (Table 3.6). No difference was found for the nutrient composition of the lactation diet when the data was taken as percentage of daily energy intake (Figure 3.18). As the maternal body weights were not measured during lactation in order to avoid disturbing lactating mothers, values per kg of body weights were unknown.

Table 3. 6 Average maternal nutrient intakes during lactation when the dietary treatment was chow diet

	CC (n=6)		CO (n=6)		OC (n=5)		OO (n=4)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Energy intake (KJ/day)	741.92	27.17	661.81*	4.48	676.38	25.67	649.61*	29.38
Fat intake (g/day)	5.83	0.56	5.01	0.50	5.38	0.53	4.99	0.60
Protein intake (g/day)	8.48	0.45	7.33	0.41	7.25	0.43	6.88	0.49
CHO intake (g/day)	23.19	1.46	21.92	1.31	22.43	1.38	19.98	1.57
SFA intake (g/day)	1.86	0.27	1.53	0.25	1.74	0.26	1.93	0.30
Sugar intake (g/day)	3.73	0.37	3.35	0.34	3.45	0.25	3.05	0.40
Na intake (g/day)	0.156	0.009	0.147	0.009	0.144	0.009	0.140	0.0010

Table 3.6 Average maternal nutrient intakes during lactation when the dietary treatment was chow diet. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet, CHO: Total carbohydrate, SFA: Saturated fatty acids, Na: Sodium. * indicates the significant effect of gestational cafeteria diet on nutrient intakes during lactation in comparison to gestational controls ($P<0.05$).

Figure 3. 17 Average maternal energy intakes when the dietary treatment during lactation was chow diet

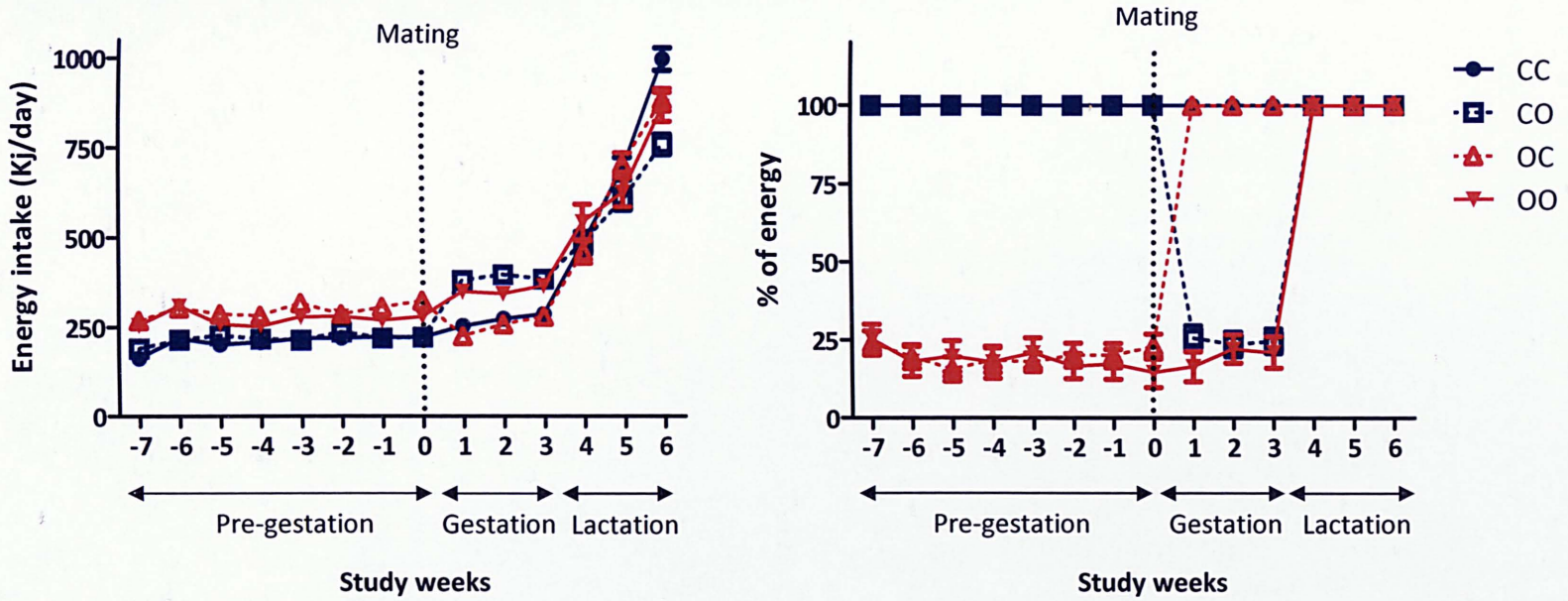


Figure 3.17 Average maternal energy intakes when the dietary treatment during lactation was chow diet. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6 or 11), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 or 12), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 or 10), OO: Cafeteria diet during pre-gestation and gestation (n=4 or 10). Study weeks between -7 and 0 represent the pre-gestational treatment. Conception is at 0. Study weeks between 1 and 3, and 4 and 6 represent pregnancy and lactation, respectively. Pre-gestational and gestational cafeteria diet significantly affected total energy intake ($P<0.001$). Gestational cafeteria diet significantly decreased the energy intake during lactation ($P<0.05$). Pre-gestational cafeteria diet groups had significantly lower intake of chow diet than controls during pre-gestational period ($P<0.001$). No effect of pre-gestational cafeteria diet intake was found on gestational chow diet intake in cafeteria diet groups during pregnancy ($P>0.05$).

Figure 3. 18 Average maternal nutrient intakes as percentage of daily energy intake when the dietary treatment during lactation was chow diet

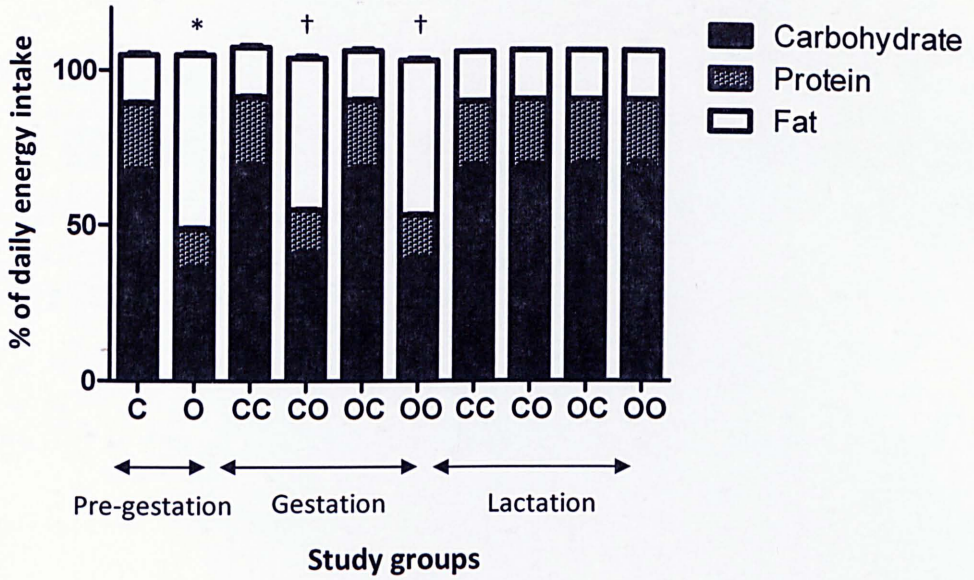


Figure 3.18 Average maternal nutrient intakes as percentage of daily energy intake when the dietary treatment during lactation was chow diet. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6 or 11), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 or 12), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 or 10), OO: Cafeteria diet during pre-gestation and gestation (n=4 or 10). Pre-gestational (* $P<0.001$) and gestational († $P<0.001$) cafeteria diet significantly increased fat and significantly lowered carbohydrate and protein intakes when the data was expressed as percentage of daily energy intake.

3.4.3.3. Cafeteria Diet During Lactation

All of the animals increased their energy intake during lactation (Figure 3.19). When the dietary treatment was cafeteria diet, the energy intake of the mothers which had cafeteria diet during pre-gestational period (OC and OO) was found to be significantly decreased than pre-gestational control animals during lactation ($P<0.05$) (Table 3.7). Cafeteria diet intake during lactation was not influenced by gestational cafeteria diet feeding ($P>0.05$) (Figure 3.19). The nutrient composition analysis showed that this was reflected in a decreased protein intake (percentage of energy) ($P<0.05$), but none of the other nutrients, including fat, altered differently (Figure 3.20). As the maternal body weights were not measured during lactation to avoid disturbing lactating mothers, values per kg of body weights were unknown.

When compared to the rats that were fed by chow diet during lactation (Table 3.6), rats fed cafeteria diet during lactation (Table 3.7) had significantly higher fat, saturated fat and sugar intakes but significantly lower protein and carbohydrate intakes ($P<0.05$ for all). However energy intakes did not differ significantly between chow and cafeteria diet fed animals during lactation (Tables 3.6 and 3.7).

Table 3. 7 Average maternal nutrient intakes when the dietary treatment during lactation was cafeteria diet

	CC (n=5)		CO (n=6)		OC (n=5)		OO (n=6)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Energy intake (KJ/day)	744.03	29.99	712.25	27.76	667.24*	27.76	632.61*	27.76
Fat intake (g/day)	8.75	0.36	7.89	0.33	7.86*	0.33	7.41*	0.33
Protein intake (g/day)	7.04	0.31	6.47	0.28	5.38*	0.28	5.71*	0.28
CHO intake (g/day)	18.42	1.20	18.25	1.11	17.23	1.11	15.97	1.11
SFA intake (g/day)	3.37	0.18	2.87	1.63	3.01	0.16	2.72	0.16
Sugar intake (g/day)	5.17	0.41	4.79	0.38	6.14	0.38	5.04	0.38
Na intake (g/day)	0.206	0.008	0.187	0.007	0.176*	0.007	0.166*	0.007

Table 3.7 Average maternal nutrient intakes when the dietary treatment during lactation was cafeteria diet. Data is shown as mean±SEM. C: Chow diet, O: Cafeteria diet, CHO: Total carbohydrate, SFA: Saturated fatty acids, Na: Sodium. * indicates the significant effect of pre-gestational cafeteria diet on nutrient intakes during lactation in comparison to pre-gestational controls ($P<0.05$).

Figure 3. 19 Average maternal energy intakes when the dietary treatment during lactation was cafeteria diet

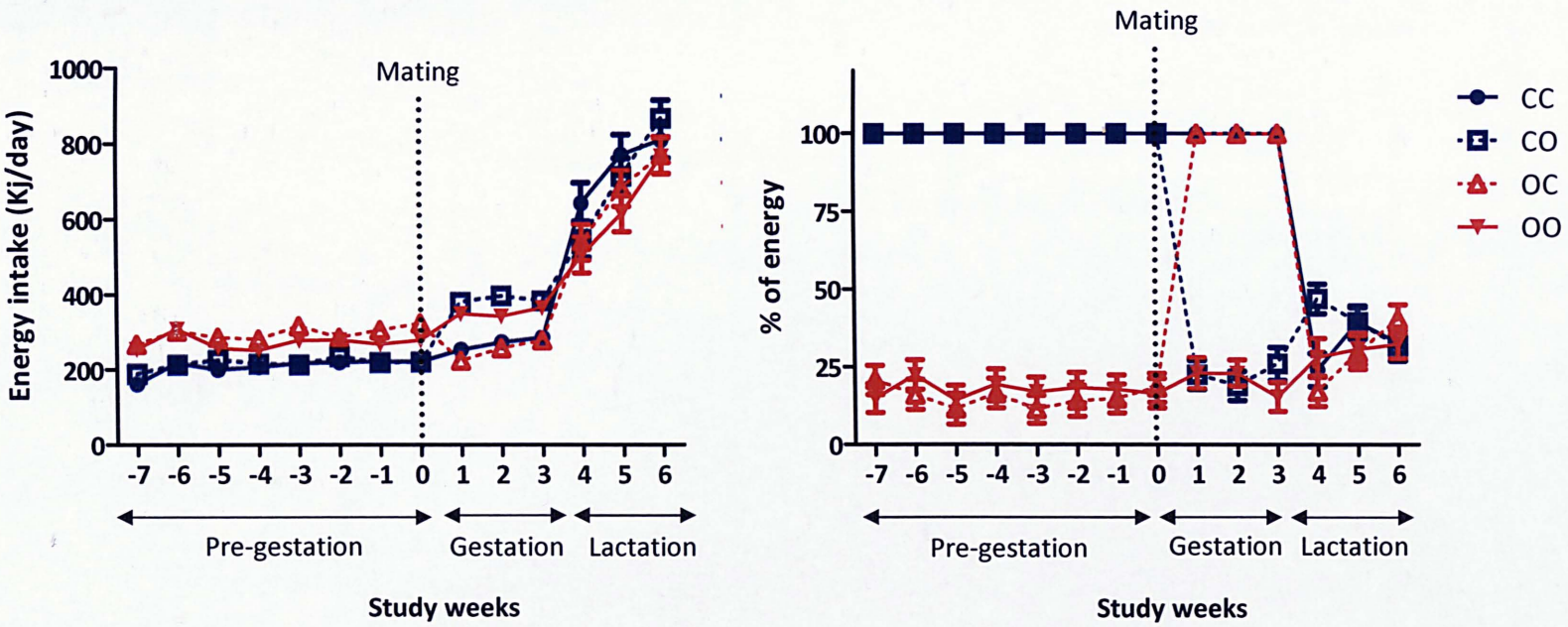


Figure 3.19 Average maternal energy intakes when the dietary treatment during lactation was cafeteria diet. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=6 or 11), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 or 12), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 or 10), OO: Cafeteria diet during pre-gestation and gestation (n=4 or 10). Study weeks between -7 and 0 represent the pre-gestational treatment. Conception is at 0. Study weeks between 1 and 3, and 4 and 6 represent pregnancy and lactation, respectively. Pre-gestational and gestational cafeteria diet significantly affected total energy intake ($P<0.001$). Pre-gestational cafeteria diet significantly decreased the energy intake during lactation ($P<0.05$). Pre-gestational or gestational cafeteria diet intake did not influence the chow diet intake in cafeteria diet groups during pregnancy or lactation ($P>0.05$).

Figure 3. 20 Average maternal nutrient intakes as percentage of daily energy when the dietary treatment during lactation was cafeteria diet

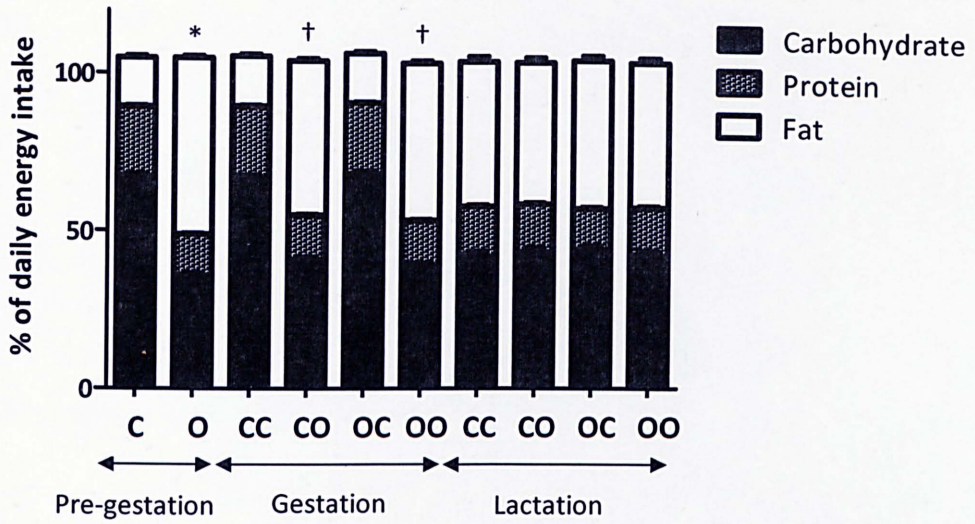


Figure 3.20 Average maternal nutrient intakes as percentage of daily energy intake when the dietary treatment during lactation was cafeteria diet. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 or 11), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 or 12), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 or 10), OO: Cafeteria diet during pre-gestation and gestation (n=6 or 10). Pre-gestational (* $P<0.001$) and gestational ($P<0.001$) cafeteria diet significantly increased fat and significantly lowered carbohydrate and protein intakes when the data was expressed as percentage of daily energy intake. Pre-gestational cafeteria diet significantly lowered the protein intake during lactation ($P<0.05$).

3.5 Discussion

The nutritional environment encountered during prenatal life is crucial in determining the future health status of the fetus. In this context, several animal studies have revealed the adverse programming effects of maternal obesity and high-fat diet on the growth and development of the fetus (*Taylor et al., 2005, Samuelsson et al., 2008, Bayol et al., 2008).* However, in such studies the effects of maternal adiposity and the diet itself on the growth and development of the fetus were combined. Identifying the initial triggering mechanisms to elucidate the underlying processes that lead to the development of the observed offspring phenotype in these studies is important. In this study, our main objective was to separate the influence of diet and maternal adiposity by introducing a pre-gestational dietary protocol (C or O) which allowed us to create four different dietary treatments (CC, CO, OC and OO) by pregnancy. We suggest that the group CO represents a model for assessing only the effects of cafeteria diet during pregnancy whereas the group OC represents a model for assessing the effects of cafeteria diet induced maternal adiposity that is independent from cafeteria diet during pregnancy. The study successfully demonstrated the differential effects of diet and obesity without influencing reproductive abilities of the dams.

The dietary composition of cafeteria diet is thought to reflect the non-prudent human diet *(Kant, 2004 and Crozier et al., 2006).* Rats were shown to become more obese with cafeteria diets rather than with purified high fat diets as a result of greater hyperphagia arising from the food variety and

palatablity *(Kretschmer et al., 2005).* It provides high fat diet content by avoiding the excessive intake of specific fatty acids. As well as the increased fat intake, increased sucrose and sodium were provided from natural foods by avoiding non-physiological amounts of single nutrients to the animal models. The possible variability between the food prefereneces of rats and over estimation of energy intake were identified as the common disadvantages of cafeteria diets *(Reuter, 2007).* However, in this study the variability between the food intake of the animals was shown to be minimal since the macronutrient composition of these animals within the same groups exhibited similar intakes and the evaporation rate of the foods were small. In addition to this, the available chow diet for these animals ensures the availability of essential nutrients.

Previously it has been reported that animal models with genetic, hypothalamic or diet induced obesity are relatively infertile or have lower litter size *(Rolls and Rowe, 1982 and Rolls et al., 1986, Guo et al., 1995, Buckley et al., 2005).* In order to test whether cafeteria diet induced obesity may impact upon fecundity of the animals, we performed a pilot trial with 6 weeks of pre-gestational dietary protocol. At the end of the 6 weeks, all the animals were mated successfully and carried pregnancy until the fifth day. Indeed, in our maternal trials, after 8 weeks of pre-gestational cafeteria diet the reproductive abilities and outcomes were not influenced by cafeteria diet. Litter sizes were similar between the groups in all experiments (CC: 12.3±0.5, CO: 11.7±0.5, OC: 13.0±0.45, OO: 11.3±1.3 pups per litter in the maternal trial

and CC: 10.60±0.28, CO: 10.36±0.26, OC: 12.0±0.26, OO: 10.56±0.29 pups per litter in the offspring trial). Moreover, plasma volume expansion was similar between cafeteria diet and chow diet fed groups in our pilot and main trial. It was reported that there was a strong relationship between plasma volume expansion and fetal growth both in human *(Salas and Rosso, 1998, Duvekot et al., 1995)* and rat studies *(Atherton et al., 1982).* Our data suggests maternal reno-vascular adaptation to pregnancy was not compromised by cafeteria diet at any stage of the pregnancy and the observed fetal outcomes were not due to this parameter.

The cafeteria diet protocol is a useful method to trigger diet induced obesity in laboratory animals. Increased adiposity and body weight were reported in other cafeteria diet studies in rats due to the variety and novelty of foods *(Rothwell and Stock, 1979, Shafat et al.,2009, Esteve et al.,1994).* Similarly, in our main maternal trial, even a short period of cafeteria diet feeding during the first 5 days of pregnancy resulted in an increase in adiposity. The group CO had 12.3 % more total body fat than the control group (CC). On the other hand, group OC had 19.1 % more total body fat when compared to CC on day 5 of pregnancy which indicated that the OC had a greater extent of maternal obesity than any pre-gestational chow diet groups. At the same time, the group OC exhibited the lowest energy intake during pregnancy in both studies. Despite this, total body weight and fat content of the OC rats were significantly higher by the end of the pregnancy than in CC controls.

Sahafat *et al.,* showed that when rats were exposed to cafeteria diet their energy intake was increased by 58 % with respect to the control group *(Shafat et al., 2009).* In our pre-gestational trials, this ratio was 12.4 % (maternal trial) and 34.0 % (maternal data obtained from offspring trial) which is relatively lower than the previous reports. This may be explained by the variety of the foods used in different studies. The novelty and the variety of the highly palatable foods used in cafeteria diet studies are thought to be the main driver of the hyperphagia and thus energy intake. In our study, we used 11 different highly palatable and highly energetic foods whereas Shafat *et al* introduced 36 different items. Nevertheless, despite their lower energy intake our cafeteria diet groups successfully developed obesity.

Decreased protein and increased fat intake were shown as typical features of cafeteria diets and the observed increase in energy intake was attributable to the increase in fat intake in cafeteria diet studies *(Llado et al., 2005, Bayol et al., 2007, Bayol et al., 2008).* The average macro-nutrient composition of our cafeteria diet foods was 8.8 % protein, 39.0 % carbohydrate and 52.1 % fat. In addition to this, chow diet was always available for the cafeteria diet fed animals. However, the average chow diet intakes of the cafeteria diet fed groups were 25.6 % of total energy intake during pre-gestation, 20.2 % during gestation and 32.0 % during lactation. Therefore, protein and carbohydrate intakes of the cafeteria diet groups remained significantly lower whereas fat intake was dramatically increased. Although 8 % protein intake has been represented as a mild low protein diet

in several studies of rat pregnancy, and it was associated with adverse programming effects on offspring *(Langley-Evans, 2004)*, Llado *et al.*, suggested that, on the whole, the quality of protein ingested by cafeteria diet animals is similar to control chow diets *(Llado et al., 1995)*.

Despite increased total body fatness and body weights, metabolic biomarkers were not greatly disturbed in cafeteria diet fed rats. Plasma cholesterol and triglyceride concentrations were similar between the groups. In parallel with our data, in other studies it has been shown that rats are resistant to develop hypercholesterolemia, unless fed a high fat-high cholesterol diet *(Chiang et al., 1998, Buettner et al., 2007)*. On day 20 of gestation the groups CO and OC showed a tendency to have 48.8 % and 41.6 % higher plasma non-fasting glucose concentrations than group CC, respectively. Interestingly, from day 5 to day 20 of pregnancy plasma non-fasting glucose concentrations decreased in all of the groups except the group CO. Similar increases in plasma glucose concentrations in dams fed high-fat diet during pregnancy were reported in other studies *(Cerf et al., 2005 and Srinivasan et al., 2006)*. In some studies however, plasma glucose concentrations were similar despite over 19 days of high-fat diets *(Tamashiro et al., 2009, Guo and Jen, 1995)*. It is clear that the nutrient composition and timing of the high-fat diets used in different studies may vary largely and this may therefore influence metabolic parameters differentially.

Exposure to excess metabolites during intrauterine life was considered as an important mechanism for fetal programming *(Dabalea, 2007)*. In

humans, maternal hyperglycaemia was reported to cause an increase in fatty streak formation in fetal artery and an accelerated progression of atherosclerosis during childhood *(Napoli et al., 1999).* The association between maternal hypercholesterolaemia and fetal programming were also shown in a rodent model *(Napoli et al., 2002).* Male offspring of mice that were fed a diet rich in cholesterol exhibited greater lesions in the aortic origin which was associated with genetic programming. Similarly, offspring of mothers with impaired glucose tolerance were shown to be more likely to develop type 2 diabetes *(Silverman et al., 1995)* and that transient neonatal exposure to hyperinsulinaemia was shown to be responsible for permanent programming of the obese phenotype *(Harder et al., 1999, Franke et al., 2005).* The lack of gross disturbance in lipid and glucose metabolism of cafeteria diet fed dams in our study may suggest the possibility that offspring of these pregnancies were not likely to be programmed by maternal hyperlipidemia or hyperglycaemia.

In our main maternal trial, the pre-gestational cafeteria diet significantly lowered the birth weight of offspring. Switching the pre-gestational cafeteria diet group to chow diet by the beginning of the pregnancy (group OC) could not rescue the fetus from this effect. The growth restriction effects of maternal obesity in this trial were not thought to be mediated by placental growth restriction since the placental weight was not influenced by pre-gestational diet. This data suggested that the maternal obesity rather than cafeteria diet itself could be responsible for the altered

intra-uterine growth in these pups up to day 20 of gestation. However, in our offspring trial, birth weight data showed not only the decreasing effect of the pre-gestational cafeteria diet consistently but also the effect of the pregnancy diet since the offspring of the animals that were fed by cafeteria diet during gestation had significantly heavier birth weights. This may imply the individual effect of the diet as cafeteria diet feeding triggers a rapid growth during the last days of pregnancy. Most of the rat studies in literature exhibited no effect of gestational high-fat diets on the birth weights of offspring *(Buckley et al.,2005, Cerf et al., 2005, Gregersen et al., 2005, Tamashiro et al., 2009, Chen et al., 2008, Ferezou-Viala et al., 2007, Srinivasan et al., 2006, Dyrskog et al., 2005, Guo and Catherine, 1995,).* Similarly Bayol *et al* did not observe a change in the birth weights of offspring of cafeteria diet fed dams during gestation only *(Bayol et al., 2005 and Bayol et al.,2007).* Howie *et al* reported a decreased birth weight in offspring of dams that were fed by 45 % fat diet, and this was observed in offspring of the dams which had high-fat diet during pregnancy only, or pre-gestation and during pregnancy. They also noted a further significant reduction in birth weight in offspring of the dams which were fed by high-fat diet only during gestation, which is inconsistent with our data *(Howie et al., 2009).* Similar reductions in birth weight were shown in offspring of protein restricted dams as a result of intrauterine growth retardation *(Fernandez-Twinn et al., 2005, Ozanne et al., 2004, Langley-Evans and Sculley, 2006).* Therefore, the composition of high fat diets exerts an important effect since the high fat diets used in different studies ranged

between 34 and 60 % of total energy content of the diet *(Ainge et al., 2010)* which may result in insufficient nutrients.

Interestingly, in our offspring trials, when the dietary treatment during lactation was chow diet the dams of the groups CO and OO reduced their energy intake compared to the other groups. This could be attributable to the switch from cafeteria diet during pregnancy to chow diet during lactation since the same pattern was also observed in the group OC at the beginning of pregnancy. However, when the dietary treatment was cafeteria diet during lactation, this time dams of the groups OC and OO also reduced their energy intakes with respect to the other groups. Previously, it has been reported that rats fed a diet with high fat concentration produce larger amounts of milk with a higher lipid concentration, than rats fed a lower fat diet *(Del Prado et al., 1999)*. At the same time, the change in the composition of the milk of the cafeteria diet dams was shown to be modified by pre-existing obesity during pregnancy *(Rolls et al., 1982)*. The protein content of the milk was reduced while the fat and energy contents were increased. Considering the OC and OO represented the heaviest groups in our trials, the reduced energy intakes in these groups when the dietary treatment was cafeteria during lactation may be explained by the protective modification of the mothers. Hamosh *et al.*, showed in rats that the increase in fat depots during pregnancy ceased during lactation, and uptake and mobilization of lipids increased into mammary tissue *(Hamosh et al., 1970)*. Since maternal body weights and adiposity were not measured in that stage of our study, this remains unclear.

In conclusion, the data in this chapter demonstrated a novel model to assess the individual effects of maternal obesity and cafeteria diet on maternal metabolism and fetal outcomes. Cafeteria diet feeding for 8 weeks during pre-gestational period and/or during pregnancy resulted in profound obesity without impacting upon the reproductive abilities of the rats. Differential effects of maternal obesity and cafeteria diet during pregnancy were exhibited for fetal growth. More importantly, the interactions between early development and later nutritional exposure was shown in several studies in offspring with altered birth weight *(Yajnik, 2000, Jaquet et al., 2005, Kensara et al., 2005).* Growth restriction during intrauterine life and subsequently catch up growth during post natal life was associated with increased adiposity and insulin resistance in rats *(Bieswal et al., 2006, Tarry-Adkins et al., 2009).* On the other hand, macrosomic offspring of hyperglycaemic mothers were also shown to be prone to development of glucose intolerance and obesity later in life *(Khan, 2007).* Therefore, it is critically important to investigate the adverse effects of maternal obesity and cafeteria diet feeding on growth and development of offspring with the implications of long term effects during post-natal life.

4.0 THE EFFECTS OF MATERNAL CAFETERIA DIET AND POSTNATAL CHOW DIET ON GROWTH, DEVELOPMENT AND GLUCOSE HOMOEOSTASIS OF THE OFFSPRING

4.1 Introduction

The risk of adulthood obesity is more than doubled in the children of obese parents *(Whitaker et al., 1997).* This relationship is largely assumed to stem from genetics or shared environment. The mechanistic link between maternal and offspring obesity is however largely uncharacterized. In the light of human and animal studies it is now widely recognized that many nutritional exposures throughout pregnancy and lactation may influence the growth and development of the offspring *(Hales and Barker, 1992, Ozanne et al., 1992, Guo and Jen, 1995).* In today's Western society, the environmental exposures of mothers which impact on their offspring are more likely to be over-nutrition and excess intake of processed foods that are rich in fat, sucrose and salt and poor in essential vitamins, minerals and fibre. Such an environment establishes adverse intrauterine conditions by inducing maternal obesity, hyperglycemia and other related complications that may consequently influence the future health status of the fetus *(Wang et al.,2000, Hillier et al., 2007).*

Previously, the adverse effects of a maternal cafeteria diet on rat offspring have been assessed in several studies by Bayol *et al (Bayol et al., 2010, Bayol et al., 2008, Bayol et al., 2005).* These studies showed that

maternal cafeteria diet in pregnancy and lactation may contribute to non-alcoholic fatty liver disease, adiposity, hyperlipidemia, early onset of hyperglycemia and impaired skeletal muscle. In addition to this, a risk of poor glycaemic control in offspring of mothers consuming a high-fat diet was reported in other rat studies *(Buckley et al., 2005, Srinivasan et al., 2006).* Samuelsson *et al.,* showed that when the offspring of high-fat fed, obese mice were weaned onto standard laboratory chow, they became hyperphagic at 4 weeks of age, and had reduced glucose tolerance at 6 months of age *(Samueslsson et al., 2008).*

The previous chapter considered the impact of cafeteria feeding in rats upon pregnancy and reproductive outcomes. It was clear that whilst rats fed cafeteria diet were able to maintain normal pregnancy, there were some effects of maternal obesity and overnutrition upon fetal growth and development. This chapter builds upon Chapter 3 by describing the effects of maternal cafeteria diet during pre-gestation and/or pregnancy on the growth and development and glucose homeostasis of offspring when the post-weaning nutritional treatment was low fat, standard laboratory chow diet. Estimated nutrient requirements were published for rodents that are largely used in biomedical research *(Knapka, 1999).* In this perspective chow diet was developed for the best attainment of particular needs such as fast growth, avoidance of obesity, longevity and reproductive performance *(Clarke et al., 1977).*

4.2 Objectives

This chapter of the study aimed to

i. Assess the effects of maternal cafeteria diet and/or maternal cafeteria diet induced obesity on the growth and nutritional habits of offspring until 3 months of age when the post-natal nutritional environment is chow diet.

ii. Explore the effects of maternal cafeteria diet and/or maternal cafeteria diet induced obesity on glucose homeostasis of offspring at 3 months of age when post-natal nutritional environment is chow diet.

iii. Compare the additional effects of chow or cafeteria diet during lactation on the previous parameters.

4.3 Materials and Methods

This chapter describes some of the data obtained from the Offspring Trial, as described in section 2.1.4. All methods are described in full in Chapter 2.

4.3.1 Biochemical Endpoints

Intraperitoneal glucose tolerance tests were performed when the offspring of rats fed cafeteria diet during different stages of pregnancy and lactation were 13 weeks old (as described in 2.2.5). Fasting and post-glucose injection plasma insulin levels were measured using a commercially available Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem Inc, USA) as described in section 2.2.3.4. Non-fasted plasma glucose, cholesterol and triglyceride

concentrations were measured through an adaptation of the glucose oxidase method and commercially available kits (as described in 2.2.3.1, 2.2.3.2 and 2.2.3.3). Expression of IRS2 and Akt2 were measured in liver as described in section 2.3.

4.3.2 Statistical Analyses

Data was analyzed using the Statistical Package for Social Sciences (version 18; SPSS, Inc., Chicago, IL, USA). Values are presented as mean ± SEM. The effects of maternal dietary treatment on offspring data were assessed using a mixed model ANOVA with fixed factors of pre-gestational diet, gestational diet and gender and random effect of litter size. Study weeks were added as a fixed factor where the nutritional data was analyzed. Body weights and energy intakes were assessed by repeated measure ANOVA. $P<0.05$ was considered as significant unless otherwise indicated. No post hoc tests were required. Differences between the groups shown in tables and figures represent main effects and interactions.

4.4 Results

4.4.1 Reproductive Outcome

All of the animals mated normally. Litter size was not affected by pre-gestational and/or gestational dietary treatment (CC: 10.60±0.28, CO: 10.36±0.26, OC: 12.0±0.26, OO: 10.56±0.29 pups per litter). Consistent with our previous data, birth weight was significantly affected by maternal diets as pre-gestational cafeteria diet groups, OC and OO, had lighter pups when

compared to CC (P<0.001) (2.3 % lower in male and % 6.1 lower in female offspring of OC with respect to CC). In addition to this, cafeteria diet in pregnancy groups, CO and OO, resulted in heavier pups when compared to CC and OC at birth (P<0.05) (6.9 % heavier in male and 3.3 % heavier in female offspring of CO with respect to CC) (Figure 4.1). Thus, the CO group had the greatest birth weight followed by group OO whereas OC had the lowest birth weight for both genders (Figure 4.1).

Figure 4. 1 Birth weights of male and female offspring

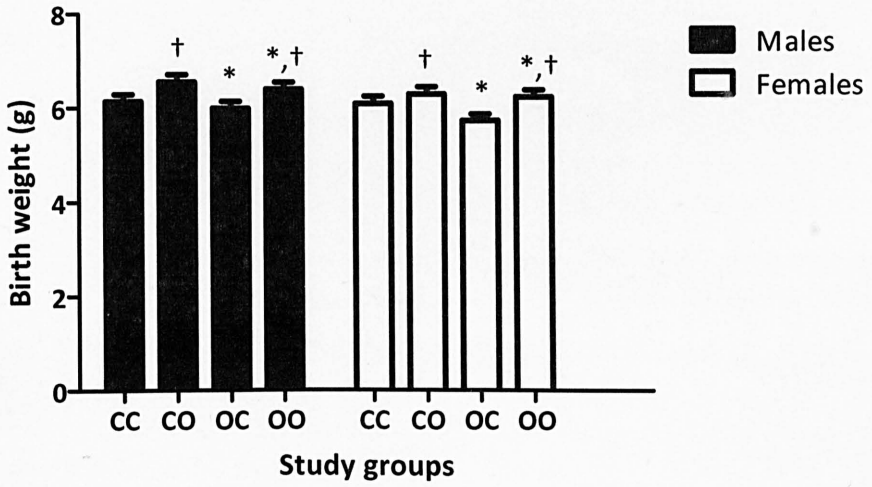


Figure 4.1 Birth weights of male and female offspring. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. * indicates the significant effect of pre-gestational cafeteria diet on birth weight of offspring in comparison to pre-gestational chow (P<0.001). indicates the significant effect of pregnancy cafeteria diet on birth weight of offspring in comparison to controls during pregnancy (P<0.05)

4.4.2 Chow diet during lactation and post-weaning period

This section will describe observations of animals exposed to chow diet during lactation.

4.4.2.1 Growth and body composition

At the end of the first week of lactation, the effect of gestational cafeteria diet on weight persisted and the offspring of pregnancy cafeteria diet groups had significantly heavier body weights (by 8.2 % for CO males, 8.6 % for CO females and 2.1 % for OO males, 5.3 % for OO females when compared to CC, $P<0.05$). Pre-gestational cafeteria diet did not have any effect on offspring body weight at that stage ($P>0.05$) (Figure 4.2). When the pups were 2 weeks old, both pre-gestational and gestational dietary treatments were found to significantly affect body weights ($P<0.001$) (Figure 4.2). In addition to this, there was a significant interaction between pre-gestational and pregnancy diets, which indicated that the group OC had the heaviest body weight at that time, in both genders, whereas OO pups had the lowest weight ($P<0.05$) (Figure 4.2).

By the time of weaning (at 3 weeks of age), all of these effects of the maternal diets disappeared and the pups of the mothers which were fed cafeteria diet during pregnancy (CO and OO) had significantly lower body weights than the corresponding controls (CC and OC) ($P<0.05$) (Figure 4.2). This effect may be explained by the fact that mothers of the pregnancy

cafeteria diet groups (CO and OO), exhibited a lower energy intake all through the lactation when compared to gestational control groups (Section 3.4.3.2).

At the beginning of the post-weaning period, the four groups of offspring did not show any significant differences in body weights when they were kept on chow diet ($P>0.05$) (Figure 4.3). Throughout the study male offspring from pre-gestational cafeteria diet groups (OC and OO) exhibited a significant deceleration of growth relative to controls and this effect remained significant until the end of the study ($P<0.05$) (Figure 4.3). On the other hand, there were no significant differences in the body weights of female offspring from 4 weeks until the end of the study which may indicate that female offspring of OC and OO had already caught up growth by this point (Figure 4.3).

When the offspring were 13 weeks old, gonadal and peri-renal fat pads exhibited unexpected differences associated with maternal cafeteria diet. There was a significant interaction between gestational dietary treatment and gender which indicated that the female CO and OO groups had 36.1 % and 44.8 % less gonadal fat respectively than groups exposed to chow diet in pregnancy ($P<0.05$) (Table 4.1). Similarly, pre-gestational cafeteria diet (OC and OO) significantly reduced the peri-renal fat pad size by 32.8 % in OC and 23.9 % in OO males, but not females ($P<0.05$) (Table 4.1). When the organ weights were analysed in terms of percentage of body weight, brain size was found to be significantly lower in the pregnancy cafeteria diet groups (CO and OO) ($P<0.05$) (Table 4.1). Male offspring of CO had 4.2 % smaller brain mass

whereas this ratio was 8.3 % lower for OO than CC. On the other hand, this proportion was 11.5 % for CO and 5.1% for OO female offspring relative to CC females. Liver and left kidney weights were significantly reduced in CO and OC as evidenced by an interaction between pre-gestational and gestational diets ($P<0.05$) (Table 4.1). Peri-renal fat, brain, liver, right and left kidney weights were significantly different between the genders ($P<0.001$) (Table 4.5). Expressed as a percentage of body weight, females tended to have larger organs than males (Table 4.1).

Figure 4. 2 Average body weights of male and female offspring during lactation when the dietary treatment was chow diet

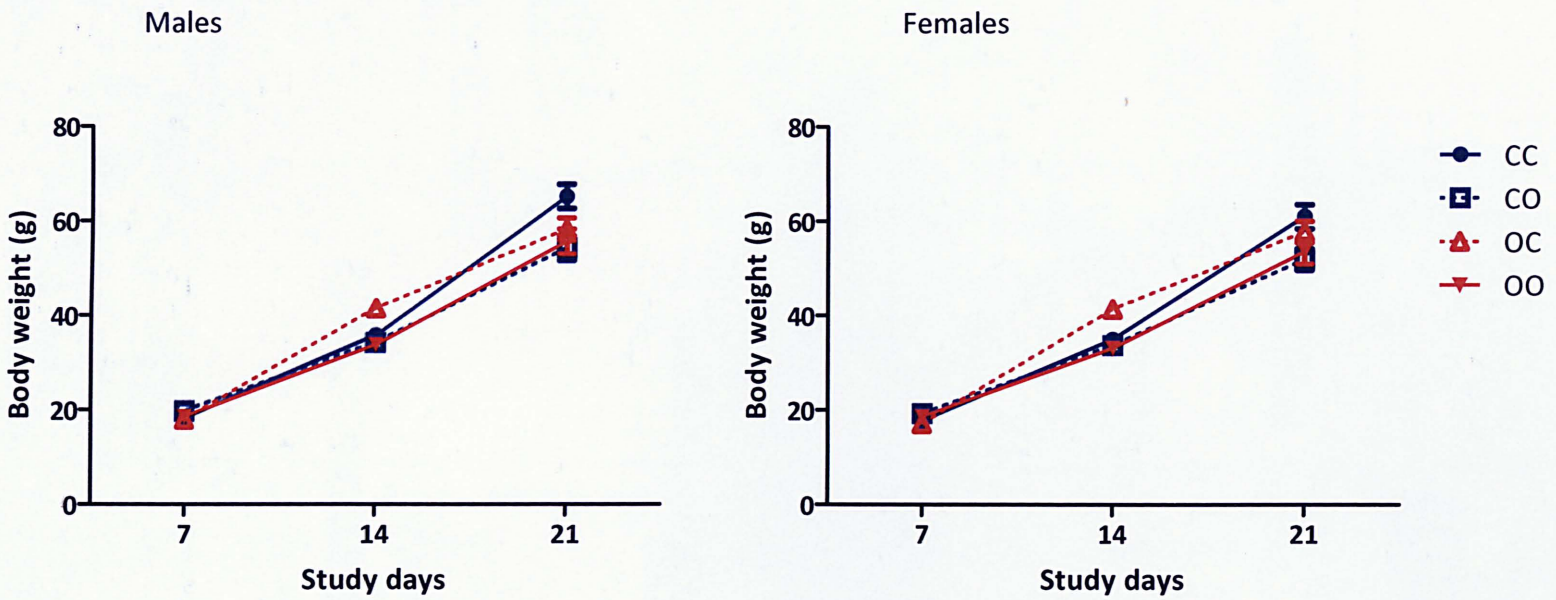


Table 4.2 Average body weights of male and female offspring during lactation when the dietary treatment was chow diet. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All mothers received chow diet during lactation. On day 14 of suckling period offspring of the group OC had the highest body weight ($P<0.05$). On the day of weaning offspring of the groups CO and OO had significantly lower body weights than CC and OC ($P<0.05$).

Figure 4. 3 Average body weights of male and female offspring exposed to chow diet during lactation and post-weaning

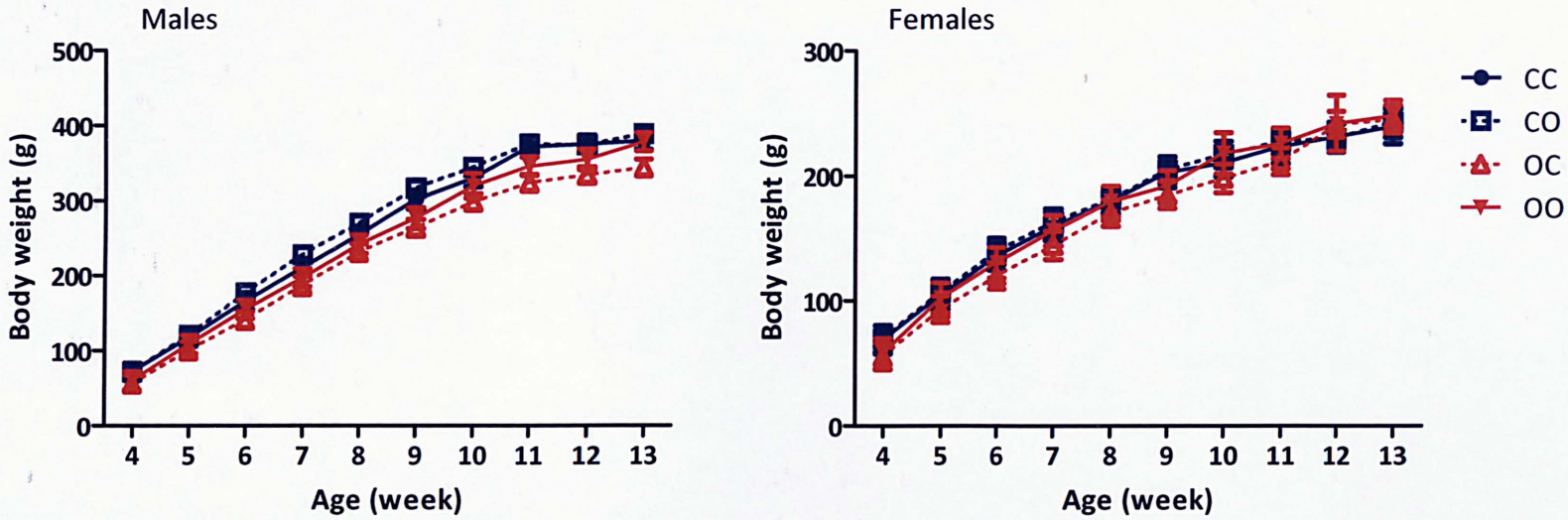


Figure 4.3 Average body weights of male and female offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to control diet during lactation and were weaned onto chow diet. The effect of gender was significant ($P<0.001$). Repeated measures ANOVA results showed that male offspring of the pre-gestational cafeteria diet groups had significantly lower body weights ($P<0.05$).

Table 4. 1 Body composition of offspring exposed to chow diet during lactation and post-weaning

		CC (n=5)		CO (n=6)		OC (n=6)		OO (n=4)	
Sex	% of body weight	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Gonadal fat	2.41	0.33	2.78	0.30	2.12	0.30	2.53	0.36
	Peri-renal fat	2.59‡	0.24	2.78‡	0.22	1.74‡*	0.22	1.97‡*	0.26
	Brain	0.48‡	0.03	0.46‡†	0.03	0.52‡	0.03	0.44‡†	0.04
	Liver	2.70‡	0.09	2.67‡¥	0.09	2.56‡¥	0.09	2.71‡	0.11
	Right kidney	0.32‡	0.02	0.29‡	0.001	0.30‡	0.001	0.29‡	0.02
	Left kidney	0.31‡	0.001	0.28‡¥	0.001	0.28‡¥	0.001	0.29‡	0.001
Female	Gonadal fat	3.35	0.33	2.14†	0.30	2.73	0.30	1.85†	0.36
	Peri-renal fat	1.54‡	0.24	1.21‡	0.22	1.29‡	0.22	1.11‡	0.26
	Brain	0.78‡	0.03	0.69‡†	0.03	0.75‡	0.03	0.74‡†	0.04
	Liver	2.82‡	0.09	2.69‡¥	0.09	2.59‡¥	0.09	3.03‡	0.11
	Right kidney	0.33‡	0.02	0.32‡	0.001	0.29‡	0.001	0.35‡	0.02
	Left kidney	0.32‡	0.001	0.29‡¥	0.001	0.29‡¥	0.001	0.34‡	0.001

Table 4.1 Body composition of offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to chow diet during gestation and were weaned onto chow diet.. * indicates the significant effect of pre-gestational cafeteria diet in comparison to pre-gestational controls ($P<0.001$). † indicates the significant effect of gestational cafeteria diet in comparison to controls during gestational period ($P<0.05$). ‡ indicates the significant effect of gender difference ($P<0.001$). ¥ indicates the significant interaction between pre-gestational and gestational diets ($P<0.05$).

4.4.2.2 Nutrient intakes

In parallel with the body weight and body composition data, pre-gestational and/or pregnancy cafeteria diet did not result in a marked increase in the food intake of the offspring when the data was expressed as average energy intake of the 13 weeks. However, interestingly, a significant interaction between gestational diet and gender was found when the data was expressed as kj consumed per kg of body weight to eliminate the confounding effect of body weight changes over study period. Female offspring of the rats fed cafeteria diet during pregnancy (CO and OO) consumed more chow than control animals, resulting in higher energy and nutrient intakes ($P<0.05$) (Table 4.2). When analysed as average weekly intakes, gender and age significantly influenced energy intakes but there was no effect of dietary group (Figures 4.4-4.7).

4.4.2.3 Circulating lipids

Plasma cholesterol and triglyceride concentrations were not dramatically affected by maternal diets. Offspring from the pre-gestational cafeteria diet groups had significantly reduced cholesterol levels relative to pre-gestational chow diet offspring ($P<0.05$) (Figure 4.8). Male offspring of OC had 21.6 % and OO had 4.1 %, female offspring of OC had 5.4 % and OO had 25.8 % lower cholesterol concentration with respect to CC. Males had significantly higher triglycerides than females ($P<0.001$) (Figure 4.8).

Table 4. 2 Average nutrient intakes of offspring exposed to chow diet during lactation and post-weaning

		Post-weaning period							
		CC (n=5)		CO (n=6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Males	Energy intake								
	(KJ/day/kg)	1418.49	104.99	1386.20	75.20	1664.03	101.83	1436.56	108.36
	(KJ/day)	311.74*	14.77	323.82*	11.27	295.99*	14.73	287.68*	15.64
	Fat intake								
	(g/day/kg)	6.08	0.46	5.95	0.32	7.01	0.43	6.08	0.47
	(g/day)	1.32*	1.03	1.38*	0.05	1.25*	0.06	1.18*	0.07
	Protein intake								
	(g/day/kg)	18.14	1.45	18.61	1.02	22.10	1.39	19.14	1.52
	(g/day)	3.89*	0.21	4.26*	0.16	3.86*	0.21	3.67*	0.22
	CHO intake								
	(g/day/kg)	56.71	4.22	57.06	3.03	66.59	4.11	57.84	4.49
	(g/day)	12.72*	0.63	13.19*	0.47	11.91*	0.62	11.47*	0.68
Females	Energy intake								
	(KJ/day/kg)	1488.00	102.68	1672.64†	87.21	1444.29	85.01	1722.94†	109.97
	(KJ/day)	245.02*	14.75	250.32*	12.81	235.16*	12.65	267.19*	15.67

Table 4.2 Average nutrient intakes of offspring exposed to chow diet during lactation and post-weaning (continued)

		Post-weaning period							
		CC (n=5)		CO (n=6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Females	Fat intake								
	(g/day/kg)	6.21	0.43	7.02†	0.37	6.08	0.36	7.27†	0.46
	(g/day)	1.03*	0.06	1.05*	0.05	0.99*	0.05	1.13*	0.07
	Protein intake								
	(g/day/kg)	19.57	1.40	22.15†	1.19	19.19	1.16	22.83†	1.50
	(g/day)	3.19*	0.21	3.28*	0.18	3.07*	0.18	3.49*	0.22
	CHO intake								
	(g/day/kg)	51.63	4.11	67.81†	3.50	57.39	3.39	69.32†	4.42
	(g/day)	8.88*	0.62	10.16*	0.54	9.44*	0.53	10.80*	0.65

Table 4.2 Average nutrient intakes of offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All offspring were exposed to chow diet during lactation and were weaned onto chow diet. * indicates the significant effect of gender difference on nutrient intakes ($P<0.001$). † indicates the significant effect of the Interaction between gestational cafeteria diet and gender ($P<0.05$).

Figure 4. 4 Average energy intakes of male and female offspring exposed to chow diet during lactation and post-weaning

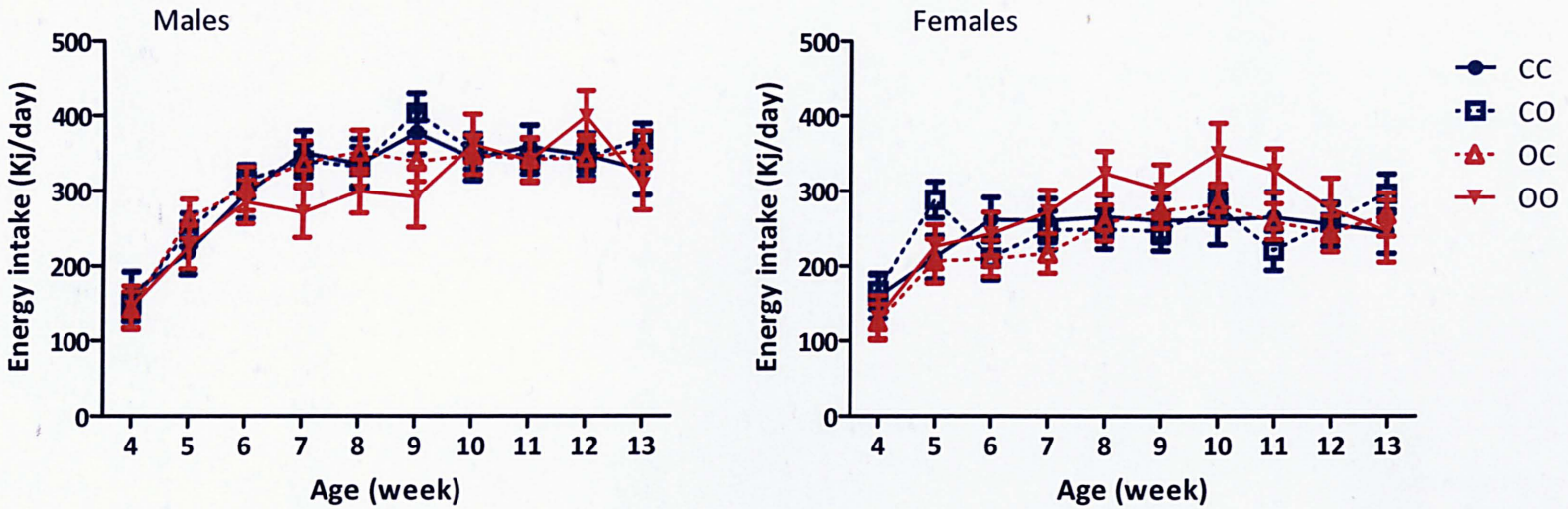


Figure 4.4 Average energy intakes of male and female offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All offspring were fed chow diet during lactation and post-weaning. The effects of gender and age were significant ($P<0.001$).

Figure 4. 5 Average protein intakes of male and female offspring exposed to chow diet during lactation and post-weaning

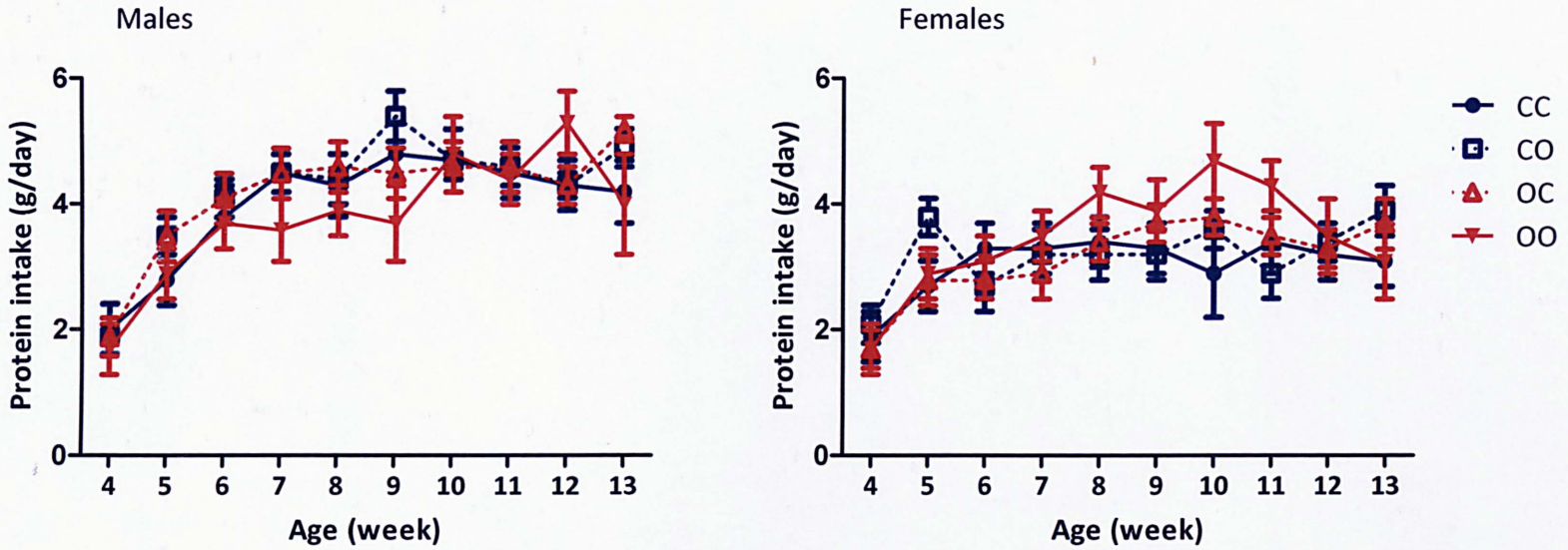


Figure 4.5 Average protein intakes of male and female offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All offspring were fed chow diet during lactation and post-weaning. The effects of gender and age were significant ($P<0.001$).

Figure 4. 6 Average fat intakes of male and female offspring exposed to chow diet during lactation and post-weaning

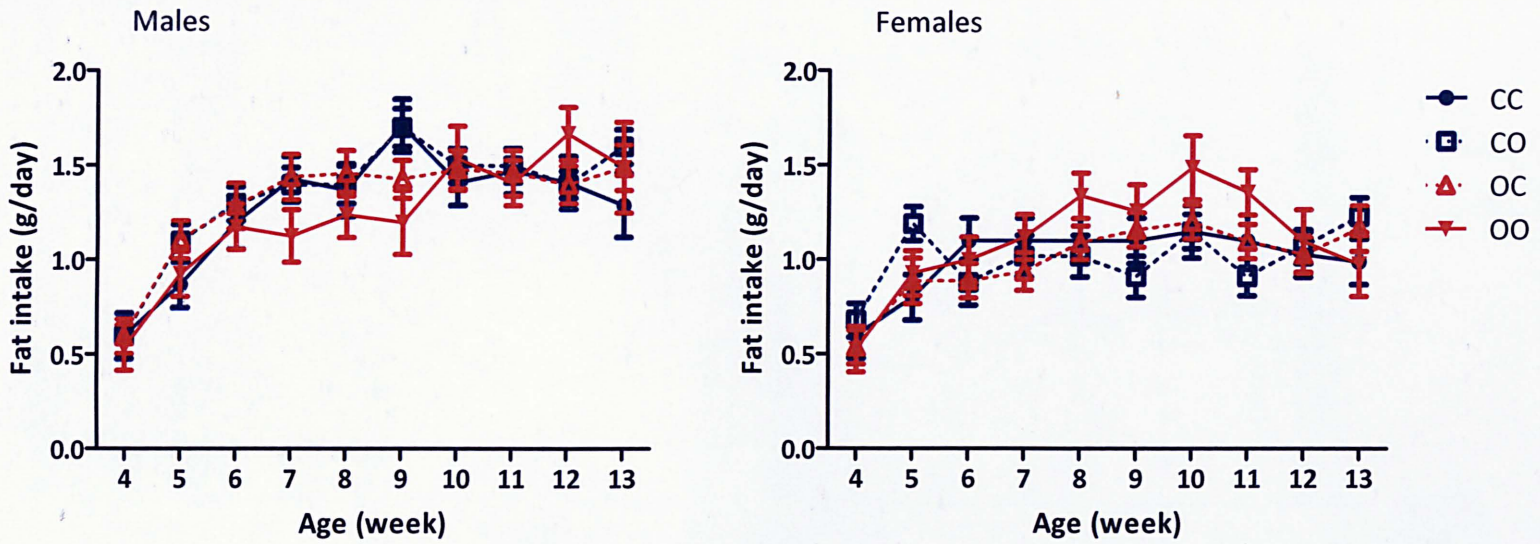


Figure 4.6 Average fat intakes of male and female offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All offspring were fed chow diet during lactation and post-weaning. The effects of gender and age were significant ($P<0.001$).

Figure 4. 7 Average carbohydrate intakes of male and female offspring exposed to chow diet during lactation and post-weaning

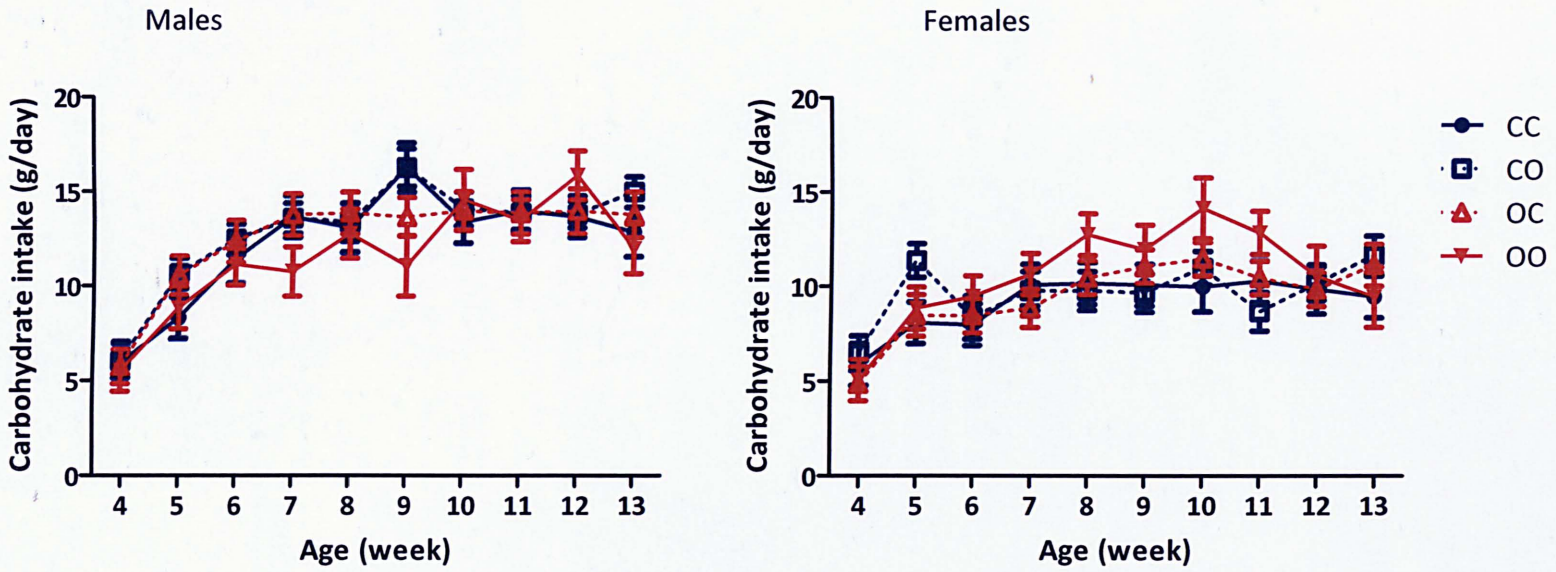


Figure 4.7 Average carbohydrate intakes of male and female offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All offspring were fed chow diet during lactation and post-weaning. The effects of gender and age were significant ($P<0.001$).

Figure 4. 8 Plasma cholesterol and triglycerides of offspring exposed to chow diet during lactation and post-weaning

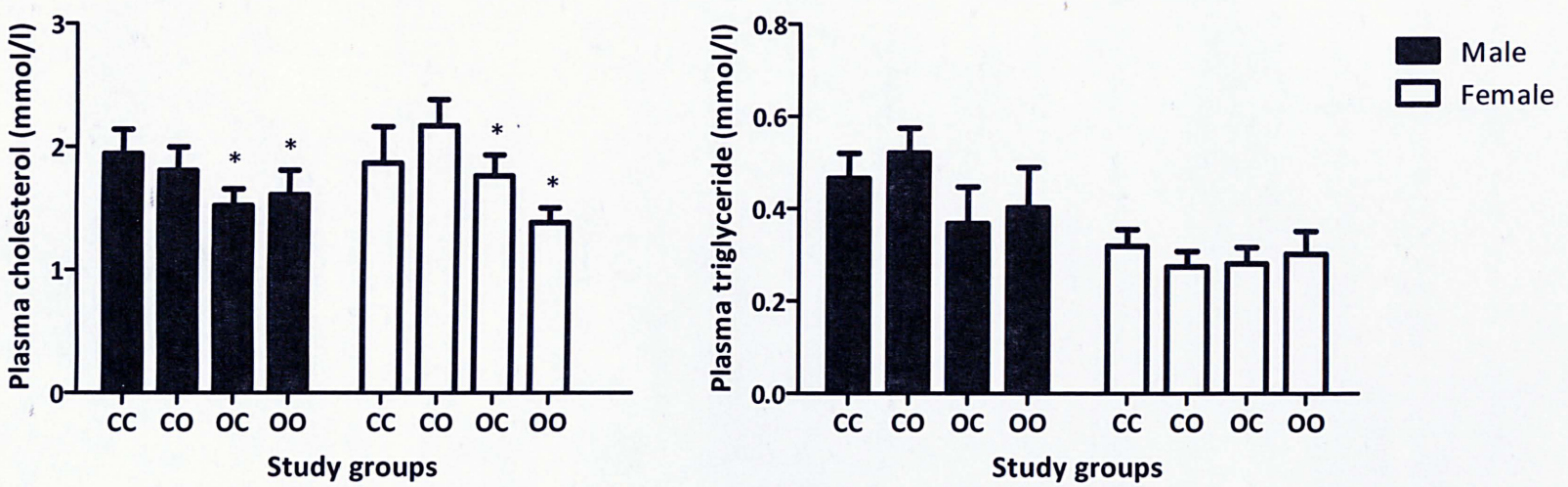


Figure 4.8 Plasma cholesterol and triglycerides of offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All offspring were fed chow diet during lactation and post-weaning period. * indicates the significant effect of pre-gestational cafeteria diet when compared to pre-gestational control groups ($P<0.05$). Sex significantly influenced plasma triglyceride concentrations ($P<0.001$).

4.4.2.4 Glucose homeostasis

Cafeteria diet significantly reduced baseline (fasting) insulin concentrations, irrespective of sex and pregnancy diet (male offspring of OC had 19.7 % and OO had 47.5 % lower, female offspring of OC had 6.4 % and OO had 41.9 % lower, $P<0.05$, Table 4.3). No difference was found in the plasma glucose concentrations at baseline and the fifth and the fifteenth minutes after the intraperitoneal injection of 2 g/kg body weight glucose (Figure 4.9). There was a significant interaction between pregnancy diet and gender such that after half an hour past injection the male offspring exposed to cafeteria diet during gestation had significantly lower plasma glucose than that exposed to chow (1.98±0.22 mg/ml for CC, 1.78±0.15 mg/ml for CO and 1.32 ± 0.18 mg/ml for OO, $P<0.05$) (Figure 4.9). In parallel with this data, in these groups the time at which peak glucose concentration occurred shifted to fifteen minutes from thirty minutes after injection (Figure 4.9). There was no influence of maternal diet on glucose concentrations at the one and two hour time points, indicating that all of the experimental groups cleared glucose over the same time as the control group (Figure 4.9).

The insulin response after half an hour post-injection exhibited a significant effect of pre-gestational cafeteria diet. Lower concentrations of plasma insulin were observed in the groups OC (10.3 % for males) and OO (29.2 % for males and 13.9 % for females) ($P<0.05$) (Table 4.3). Female offspring of OC group did not exhibit this effect. In addition to this, there was a significant interaction between pre-gestational and pregnancy diets, with

the data showing that the insulin concentrations were increased in the groups CO and OC, but male offspring of the group OC did not exhibit this effect ($P<0.05$) (Table 4.3). This effect was marked in the CO group, with a 57.1 % increase for males and 127.8 % increase for females (Table 4.3). These significant differences remained when the data was expressed as Δ insulin (the difference between baseline and thirty minute insulin concentrations) ($P<0.05$) (Table 4.3). Interestingly, area under the curve data for glucose concentrations showed a tendency for females exposed to pre-gestational cafeteria diet group (OC and OO) to have lower values than the other groups suggesting slightly more rapid glucose clearance (interaction between pre-gestational diet and gender ($P=0.08$) (Table 4.3).

The hepatic expression of genes involved in the insulin signalling pathway showed little effect of the maternal diet. Males exposed to pre-gestational cafeteria diet groups (OC and OO) had lower IRS2 mRNA expression (0.55 ± 0.14 IRS2/βactin for CC, 0.23 ± 0.03 IRS2/βactin for OC and 0.34±0.14 IRS2/βactin for OO), whilst there was no difference in AKT2 mRNA expression ($P>0.05$) (Figure 4.10). No difference was found in the expression of IRS2 and AKT2 in female animals ($P>0.05$).

Table 4. 3 Glucose tolerance data of offspring exposed to chow diet during lactation and post-weaning period

		CC (n=5)		CO (n=6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Baseline glucose (mg/ml)	0.61‡	0.03	0.78‡	0.06	0.62‡	0.07	0.70‡	0.04
	Baseline insulin (ng/ml)	0.61‡	0.16	0.77‡	0.19	0.49‡*	0.11	0.32‡*	0.06
	Insulin at 30 min (ng/ml)	2.33‡	0.61	3.66‡¥	0.62	2.09‡*	0.59	1.65‡*	0.62
	Δ insulin (ng/ml)	1.62‡	0.70	3.08‡¥	0.73	1.54‡¥*	0.50	1.30‡*	0.58
	AUC glucose (mg/ml.min)	144.06‡	10.74	130.04‡	18.53	160.59‡	26.47	129.73‡	16.22
Female	Baseline glucose (mg/ml)	0.63‡	0.02	0.69‡	0.04	0.66‡	0.04	0.64‡	0.06
	Baseline insulin (ng/ml)	0.31‡	0.07	0.31‡	0.08	0.29‡*	0.06	0.18‡*	0.05
	Insulin at 30 min (ng/ml)	1.15‡	0.40	2.62‡¥	0.30	1.79‡¥	0.37	0.99‡*	0.18
	Δ insulin (ng/ml)	1.31‡	0.19	2.20‡¥	0.41	1.43‡¥	0.32	0.78‡	0.14
	AUC glucose (mg/ml.min)	101.04‡	9.11	117.77‡	15.18	78.88‡∫	11.56	75.80‡∫	11.06

Table 4.3 Glucose tolerance data of offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to chow diet during lactation and weaned onto chow diet.. * indicates the significant effect of pre-gestational cafeteria diet in comparison to pre-gestational controls ($P<0.001$). Δ insulin: the difference between baseline and thirty minute insulin concentrations. † indicates the significant effect of pregnancy cafeteria diet in comparison to controls during gestational period ($P<0.05$). ‡ indicates the significant effect of gender difference ($P<0.001$). ¥ indicates the significant interaction between pre-gestational and pregnancy diets ($P<0.05$). ∫ indicates the tendency towards an interaction between pre-gestational diet and gender ($P=0.08$).

Figure 4. 9 Plasma glucose concentrations following intraperitoneal glucose tolerance test of offspring exposed to chow diet during lactation and post-weaning

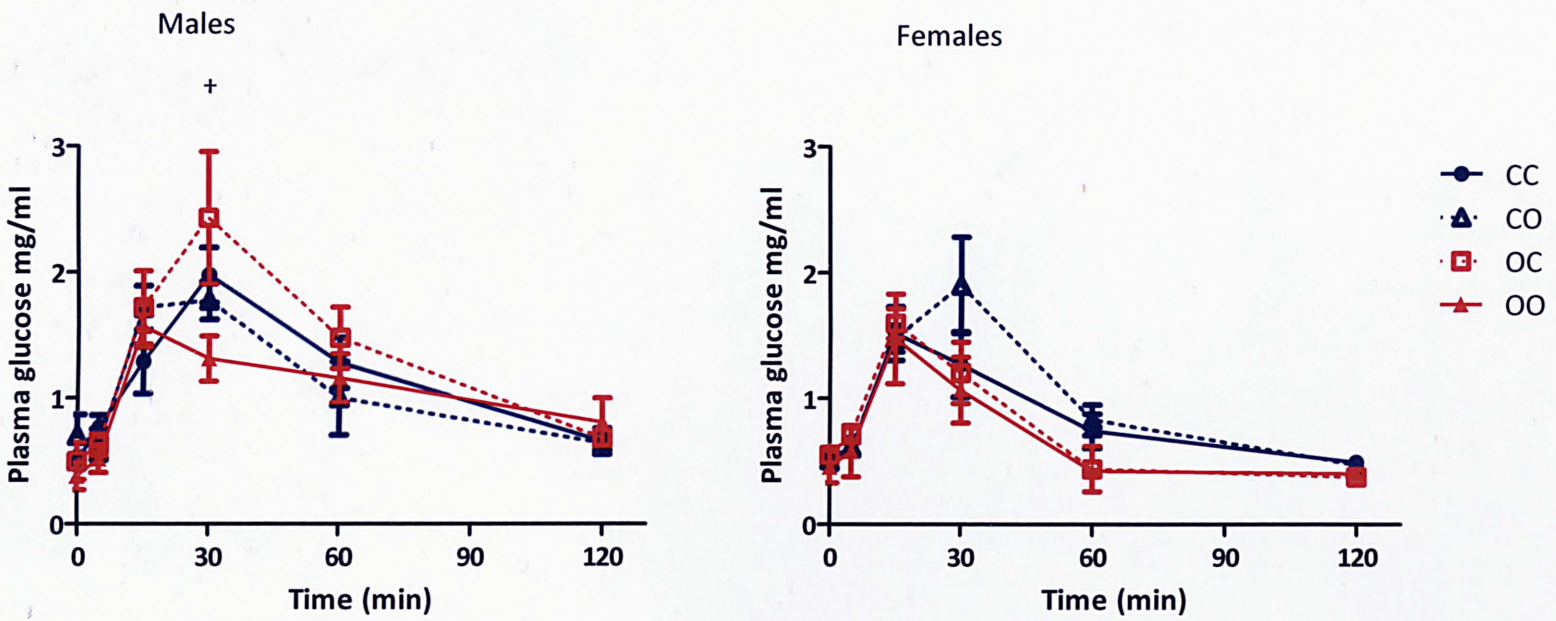


Figure 4.9 Plasma glucose concentrations following intraperitoneal glucose tolerance test of offspring exposed to chow diet during lactation and post-weaning. Data was analysed at each time point separately for the effects of gender, pre-gestational and pregnancy diets. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and post-weaning period. † indicates the significant interaction between pregnancy diet and gender ($P<0.05$). Gender effect was significantly different ($P<0.05$).

Figure 4. 10 Expression of genes in the insulin signalling pathway of offspring exposed to chow diet during lactation and post-weaning

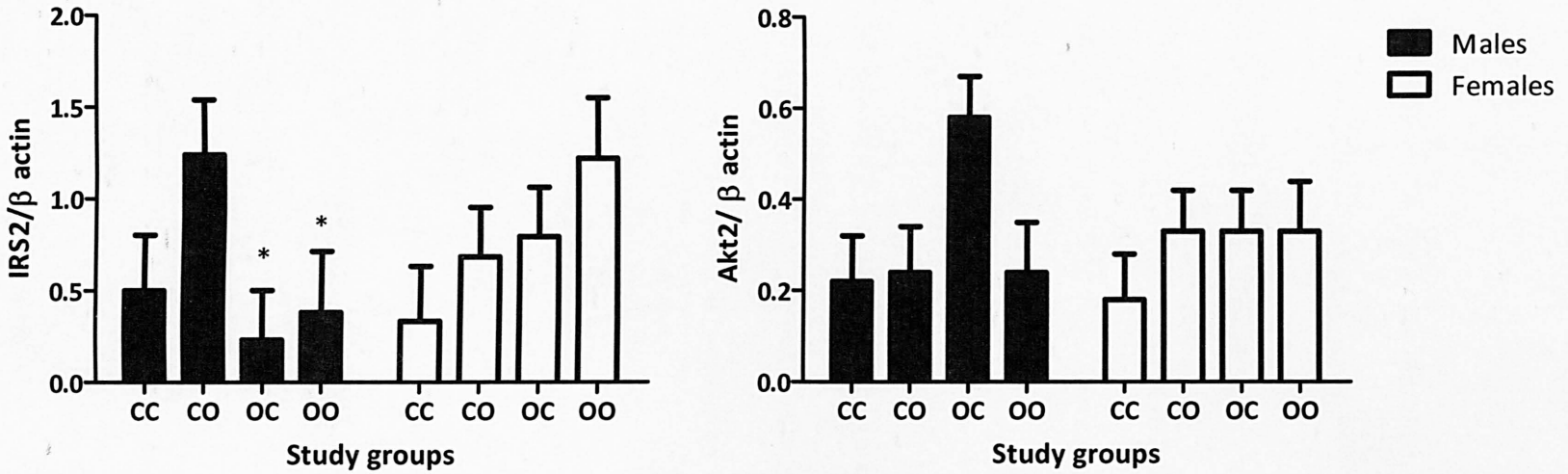


Figure 4.10 Expression of genes in the insulin signalling pathway of offspring exposed to chow diet during lactation and post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and post-weaning period. * indicates the significant interaction between pre-gestational diet and gender ($P<0.05$).

4.4.3 Cafeteria diet during lactation and chow diet during post-weaning

This section will describe observations of offspring where mothers were fed cafeteria diet during lactation.

4.4.3.1 Growth and body composition

Unlike the offspring of mothers fed chow diet during lactation, after a week of cafeteria diet during lactation, pups of the pre-gestational cafeteria diet groups (OC and OO) were of greater body weight, although they had had a lower body weight at birth ($P<0.05$) (3.9 % heavier in male offspring of OC, 10.7 % heavier in male offspring of OO and 4.4 % heavier in female offspring of OO with respect to CC) (Figure 4.11). This effect was not observed in the female offspring of OC when compared to CC. By the second week of the lactation on cafeteria diet, both pre-gestational and pregnancy diets were found to significantly influence weight and all of the groups which had been exposed to cafeteria diet at any stage of the study were heavier than the control group (for the male offspring: 19.3 % in OC, 28.1 % in CO, 29.9 % in OO and for the female offspring: 20.2 % in OC, 16.6 % in CO and 25.8 % in OO) ($P<0.001$) (Figure 4.11). At the time of the weaning this effect remained significant only for the pregnancy cafeteria diet groups except the female offspring of CO with respect to CC ($P<0.05$) (5.2 % for male offspring of CO and 3.2 % and 3.4 % for male and female offspring of OO, respectively) (Figure 4.11).

During the post-weaning period, the impact of the cafeteria diet during lactation was seen to interact with pregnancy cafeteria diet. The offspring of the mothers which were fed cafeteria diet during pregnancy (CO and OO) had significantly higher body weights throughout the study when compared to offspring of the mothers which were fed chow diet (CC and OC) ($P<0.001$) (Figure 4.12). This effect was stronger in males than in females (interaction between pre-gestational diet and gender, $P<0.05$) (372.52 ± 14.94 g in male offspring of CC versus 387.20 ± 18.14 g in male offspring of CO and 384.70 ± 7.39 g in male offspring of OO).

Despite the heavier body weights of pregnancy cafeteria diet groups, body composition data did not indicate any remarkable changes in adiposity. In parallel with our previous data, pre-gestational cafeteria diet groups (OC and OO) had significantly lower gonadal and peri-renal fat mass ($P<0.005$) (Table 4.4). Brain, liver, left and right kidney weights did not show any differences between the groups (Table 4.4).

Figure 4. 11 Average body weights of male and female offspring during lactation when the dietary treatment was cafeteria diet

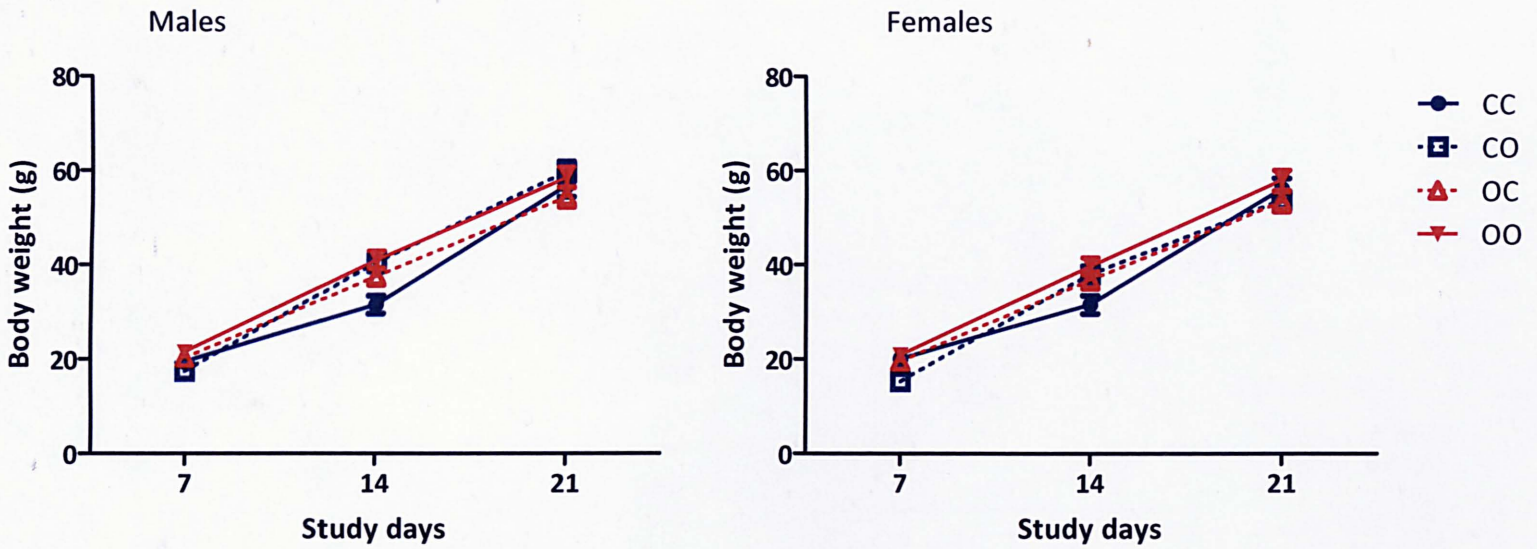


Figure 4.11 Average body weights of male and female offspring during lactation when the dietary treatment was cafeteria diet. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All animals were exposed to cafeteria diet during lactation and weaned onto chow diet. On day 7 offspring of pre-gestational cafeteria diet groups were significantly heavier than pre-gestational chow diet groups ($P<0.05$). On day 14 all of the offspring of maternal cafeteria diet groups were significantly heavier than control groups ($P<0.001$). At the end of lactation offspring of pregnancy cafeteria diet groups were significantly heavier than pregnancy chow diet groups ($P<0.05$).

Figure 4. 12 Average body weights of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

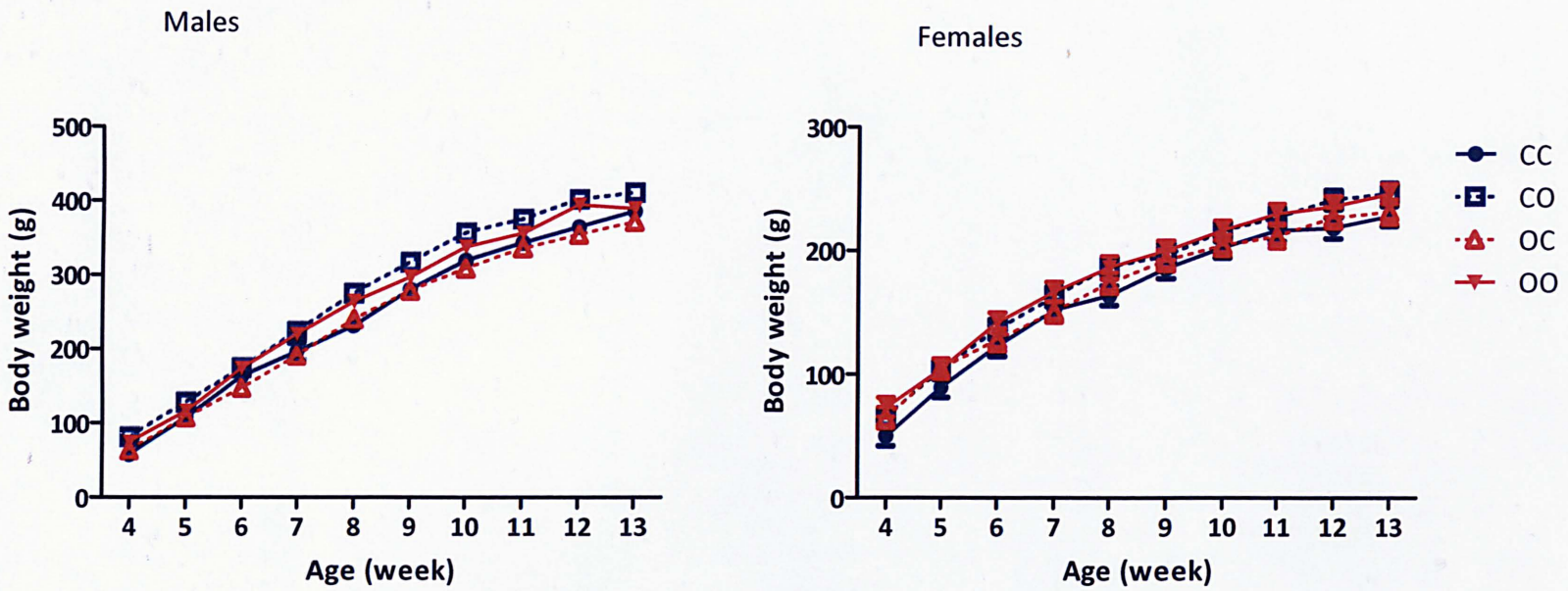


Figure 4.12 Average body weights of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet during lactation and weaned onto chow diet. The effects of gender and age were significant ($P<0.001$). Offspring of the pregnancy cafeteria diet groups had significantly higher body weights ($P<0.001$).

Table 4. 4 Body composition of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

		CC (n=5 or 6)		CO (n=4 or 6)		OC (n=5)		OO (n=5 or 6)	
Sex	% of body weight	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Gonadal fat	2.69	0.31	2.49	0.34	2.14*	0.30	2.40*	0.31
	Peri-renal fat	3.18‡	0.23	2.46‡	0.26	2.21‡*	0.23	2.26‡*	0.23
	Brain	0.47‡	0.02	0.46‡	0.02	0.49‡	0.02	0.46‡	0.02
	Liver	2.70	0.12	2.70	0.13	2.53	0.11	2.63	0.11
	Right kidney	0.28	0.001	0.29	0.02	0.31	0.001	0.30	0.001
	Left kidney	0.27	0.001	0.28	0.001	0.30	0.001	0.30	0.001
Female	Gonadal fat	3.45	0.28	2.54	0.29	2.25*	0.30	2.57*	0.28
	Peri-renal fat	1.67‡	0.21	1.55‡	0.21	1.34‡*	0.23	1.31‡*	0.21
	Brain	0.69‡	0.02	0.69‡	0.02	0.67‡	0.02	0.72‡	0.02
	Liver	2.80	0.11	2.68	0.11	2.46	0.13	2.69	0.11
	Right kidney	0.31	0.001	0.32	0.001	0.28	0.02	0.29	0.001
	Left kidney	0.29	0.001	0.32	0.001	0.30	0.001	0.30	0.001

Table 4.4 Body composition of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. * indicates the significant effect of pre-gestational cafeteria diet in comparison to pre-gestational controls ($P<0.05$). ‡ indicates the significant effect of gender difference ($P<0.001$).

4.4.3.2 Nutrient intakes

In this arm of the trial, energy intakes of the groups showed sex specific differences (Table 4.5). A significant interaction between pre-gestational diet, pregnancy diet and gender showed that males of all the groups which had cafeteria diet at any pre-natal stage of the study (CO, OC and OO) had higher energy intakes than controls ($P<0.05$, Table 4.5 and Figure 4.13). Figures 4.14, 4.15 and 4.16 show average daily protein, fat and carbohydrate intakes respectively at different stages of the study.

4.4.3.3 Circulating lipids

There was a significant interaction between pre-gestational diet, pregnancy diet and gender for plasma cholesterol concentrations ($P<0.05$) (Figure 4.17). This indicated that female offspring of the group OC had lower cholesterol than controls (2.25 ± 0.23 mmol/L in CC versus 1.74 ± 0.12 mmol/L in OC). No equivalent change was seen in male offspring of OC. No significant effect of pre-gestational or pregnancy diets was noted for plasma triglyceride concentrations ($P>0.05$) (Figure 4.17). Males had higher triglyceride concentrations than females ($P<0.05$) (Figure 4.17).

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Table 4. 5 Average nutrient intakes of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

		Post-weaning period							
		CC (n=5 or 6)		CO (n=4 or 6)		OC (n=5)		OO (n=5 or 6)	
Sex		Mean	SEM	Mean	SEM	,Mean	SEM	Mean	SEM
Males	Energy intake								
	(KJ/day/kg)	1333.79‡	110.12	1416.46‡†	113.33	1581.55‡†	103.07	1448.90‡	101.84
	(KJ/day)	286.07	13.17	319.32†	12.70	317.65†	12.03	310.16	12.11
	Fat intake								
	(g/day/kg)	5.95‡	0.33	5.82‡	0.33	6.49‡	0.30	6.16‡	0.30
	(g/day)	1.30	0.05	1.35	0.05	1.34	0.04	1.34	0.05
	Protein intake								
	(g/day/kg)	19.14‡	1.16	18.83‡	1.26	21.09‡	1.16	19.91‡	1.12
	(g/day)	4.18	0.13	4.24	0.13	4.22	0.12	4.17	0.12
	CHO intake								
	(g/day/kg)	58.05‡	3.69	57.12‡	4.02	67.88‡	3.61	60.38‡	3.57
	(g/day)	12.52	0.44	12.78	0.44	13.51	0.39	12.77	0.40
Females	Energy intake								
	(KJ/day/kg)	1662.06‡	102.57	1533.54‡	100.88	1545.15‡	101.60	1491.37‡	99.13
	(KJ/day)	249.38	12.66	248.28	12.01	226.78	11.89	229.57	12.05

Table 4.5 Average nutrient intakes of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning (continued)

		Post-weaning period							
		CC (n=5)		CO (n=6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Females	Fat intake								
	(g/day/kg)	6.96‡	0.39	6.14‡	0.39	6.53‡	0.40	5.65‡	0.38
	(g/day)	1.00	0.05	1.00	0.05	0.96	0.04	0.89	0.04
	Protein intake								
	(g/day/kg)	21.76‡	1.11	19.16‡	1.06	20.28‡	1.14	20.63‡	1.06
	(g/day)	3.20	0.13	3.12	0.12	3.04	0.12	3.11	0.12
	CHO intake								
	(g/day/kg)	66.02‡	3.53	58.86‡	3.39	61.77‡	3.61	61.79‡	3.39
	(g/day)	9.61	0.44	9.49	0.40	9.09	0.40	9.49	0.39

Table 4.5 Average nutrient intakes of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. † indicates the significant interaction between pre-gestational diet, pregnancy diet and gender ($P<0.05$). ‡ indicates the significant effect of gender ($P<0.001$).

Figure 4. 13 Average energy intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

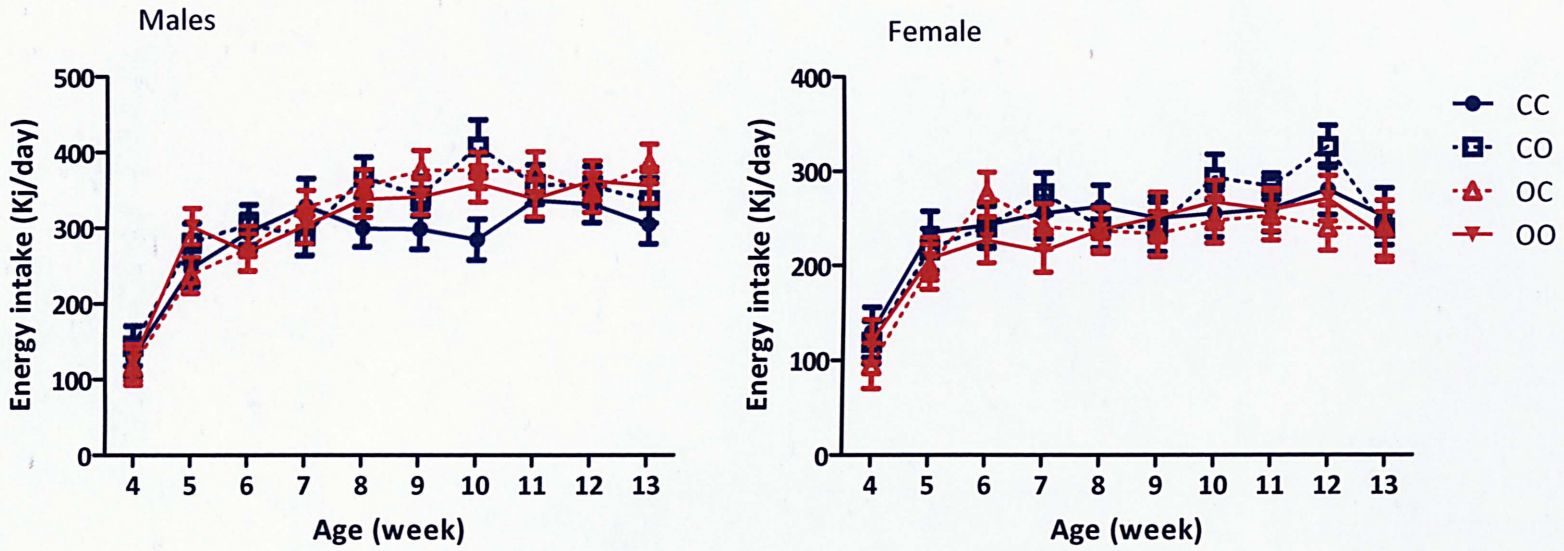


Figure 4.13 Average energy intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. There was a significant interaction between pre-gestational diet and gender ($P<0.05$). The effect of gender and age were significant ($P<0.001$).

Figure 4. 14 Average protein intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

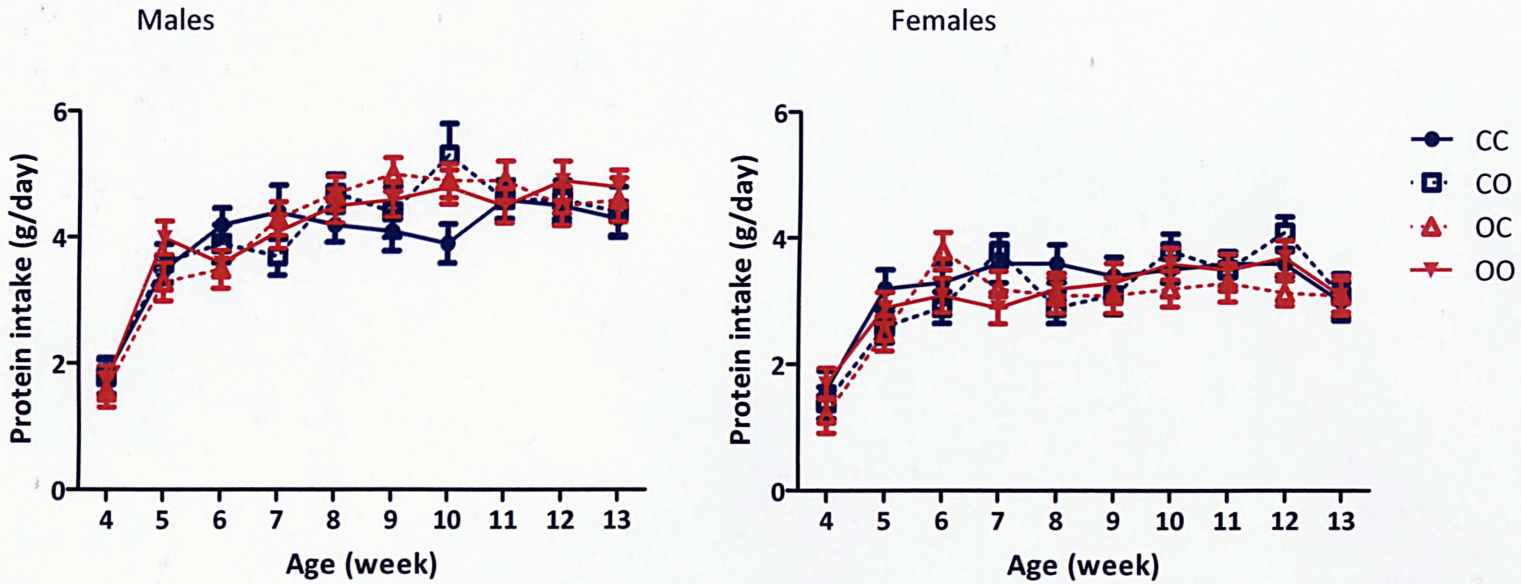


Figure 4.14 Average protein intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. The effect of gender and age were significant ($P<0.001$).

Figure 4. 15 Average fat intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

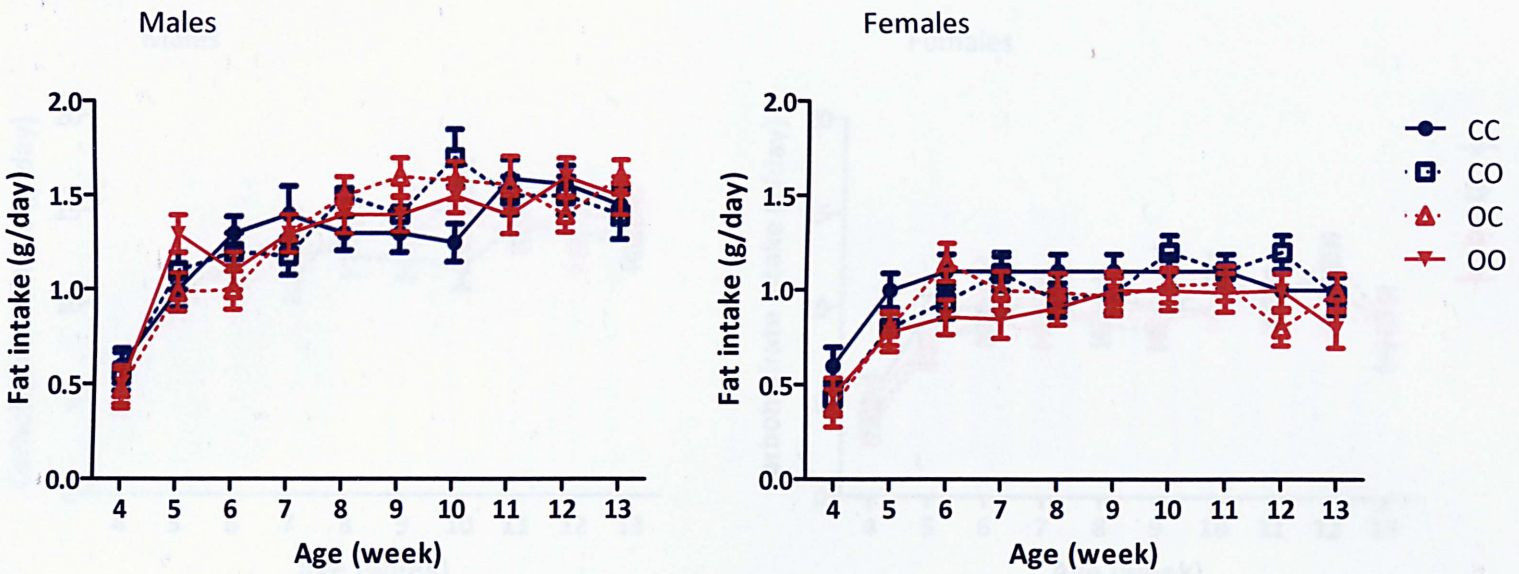


Figure 4.15 Average fat intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. The effect of gender and age were significant ($P<0.001$).

Figure 4. 16 Average carbohydrate intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

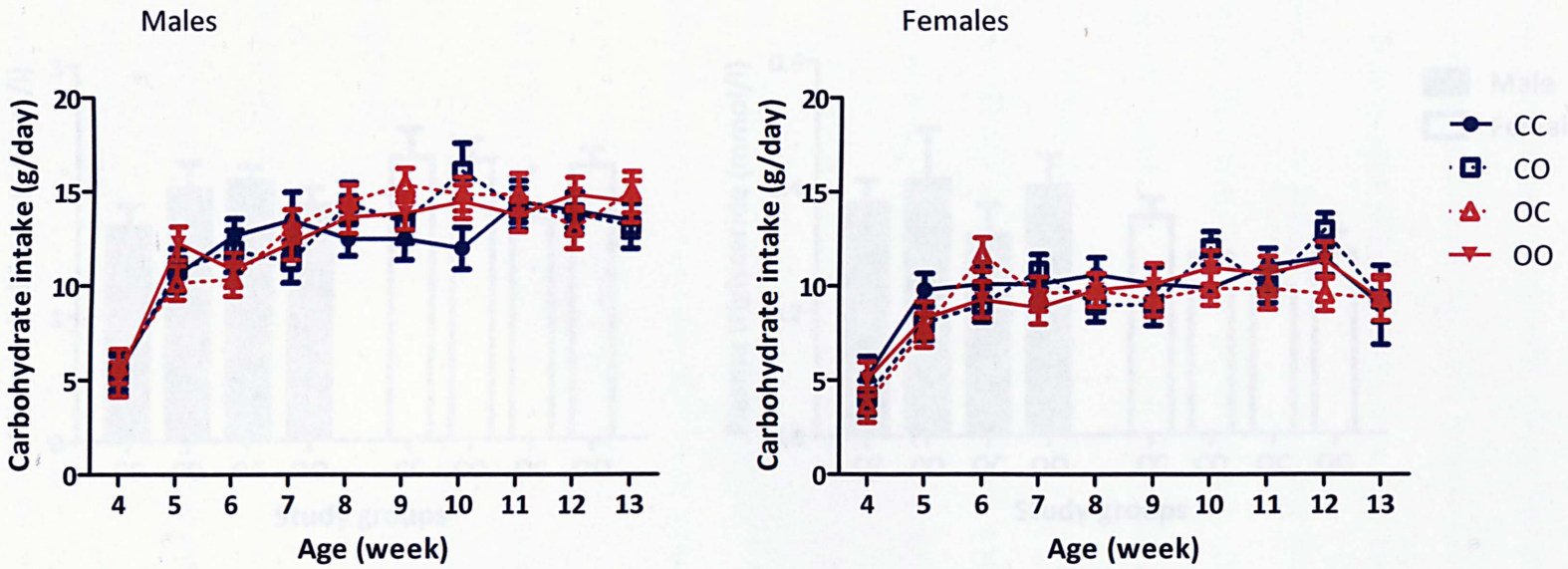


Figure 4.16 Average carbohydrate intakes of male and female offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. The effect of gender and age were significant ($P<0.001$).

Figure 4. 17 Plasma cholesterol and triglycerides of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

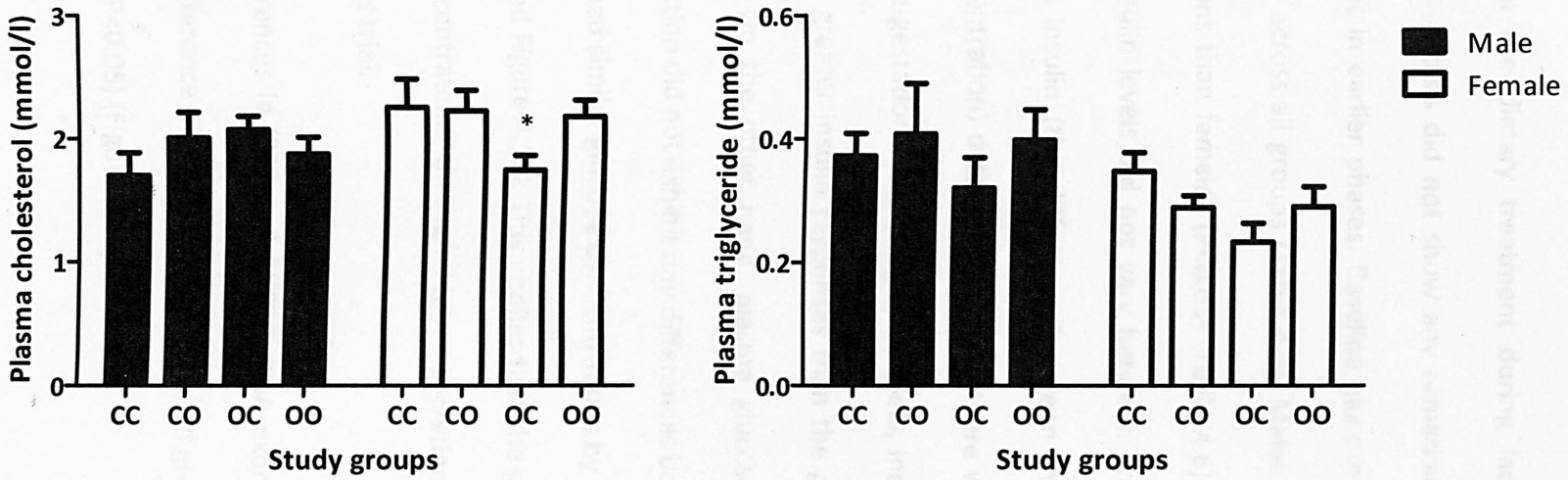


Figure 4.17 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet in the lactation period and were weaned onto chow diet. * indicates the significant interaction of pre-gestational diet, pregnancy diet and gender on plasma cholesterol concentrations ($P<0.05$). Gender significantly influenced on plasma triglyceride concentrations ($P<0.05$).

4.4.3.4 Glucose homeostasis

When the dietary treatment during lactation was cafeteria diet, glucose homeostasis did not show any remarkable disturbances ralated to cafeteria diet in earlier phases. Baseline glucose and insulin concentrations were similar across all groups (Table 4.6). Males had higher baseline insulin concentrations than females ($P<0.05$) (Table 4.6). Half an hour post-glucose injection, insulin levels did not vary between groups (Table 4.6). However, when the $\hat{\Delta}$ insulin (the difference between the baseline and 30 minute insulin concentration) data was analyzed, there was a significant interaction between pre-gestational and pregnancy diets, indicating that the groups CO and OC had greater insulin responses than the groups CC and OO ($P<0.05$) (Table 4.6). On the other hand, plasma glucose concentrations after the glucose injection did not exhibit any differences between the groups and all of the groups had similar glucose concentrations by the end of the second hour (Table 4.6 and Figure 4.18). This implies that the groups CO and OC regulated glucose concentrations through higher secretion of insulin than the other groups in this trial.

Differences in IRS2 and AKT2 mRNA expression levels did not reach statistical difference (Figure 4.19). Females had greater IRS2 mRNA expression than males ($P<0.05$) (Figure 4.19).

Table 4. 6 Glucose tolerance data of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

		CC (n=5 or 6)		CO (n=4 or 6)		OC (n=5)		OO (n=5 or 6)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Baseline glucose (mg/ml)	0.66	0.08	0.87	0.03	0.84	0.03	0.86	0.03
	Baseline insulin (ng/ml)	0.33‡	0.09	0.52‡	0.33	0.53‡	0.18	0.69‡	0.14
	Insulin at 30 min (ng/ml)	1.92‡	0.29	3.47‡	1.48	2.84‡	0.27	2.18‡	0.55
	Δ insulin (ng/ml)	1.59	0.21	2.94¥	1.18	2.26¥	0.12	1.48	0.46
	AUC glucose (mg/ml.min)	118.91	13.66	115.33	9.96	109.56	12.59	130.80	6.01
Female	Baseline glucose (mg/ml)	0.66	0.03	0.60	0.02	0.72	0.05	0.69	0.04
	Baseline insulin (ng/ml)	0.30‡	0.08	0.07‡	0.02	0.28‡	0.08	0.32‡	0.12
	Insulin at 30 min (ng/ml)	1.42‡	0.48	1.37‡	0.11	2.36‡	0.34	1.62‡	0.38
	Δ insulin (ng/ml)	1.19	0.46	1.29¥	0.09	2.22¥	0.44	1.41	0.42
	AUC glucose (mg/ml.min)	89.36	12.03	118.51	17.31	109.56	12.03	103.80	14.06

Table 4.6 Glucose tolerance data of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All the groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. Δ Insulin: the difference between baseline and thirty minute insulin concentrations. ‡ indicates the significant effect of gender ($P<0.001$). ¥ indicates the significant interaction between pre-gestational and pregnancy diets ($P<0.05$).

Figure 4. 18 Plasma glucose concentrations following intraperitoneal glucose tolerance test of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

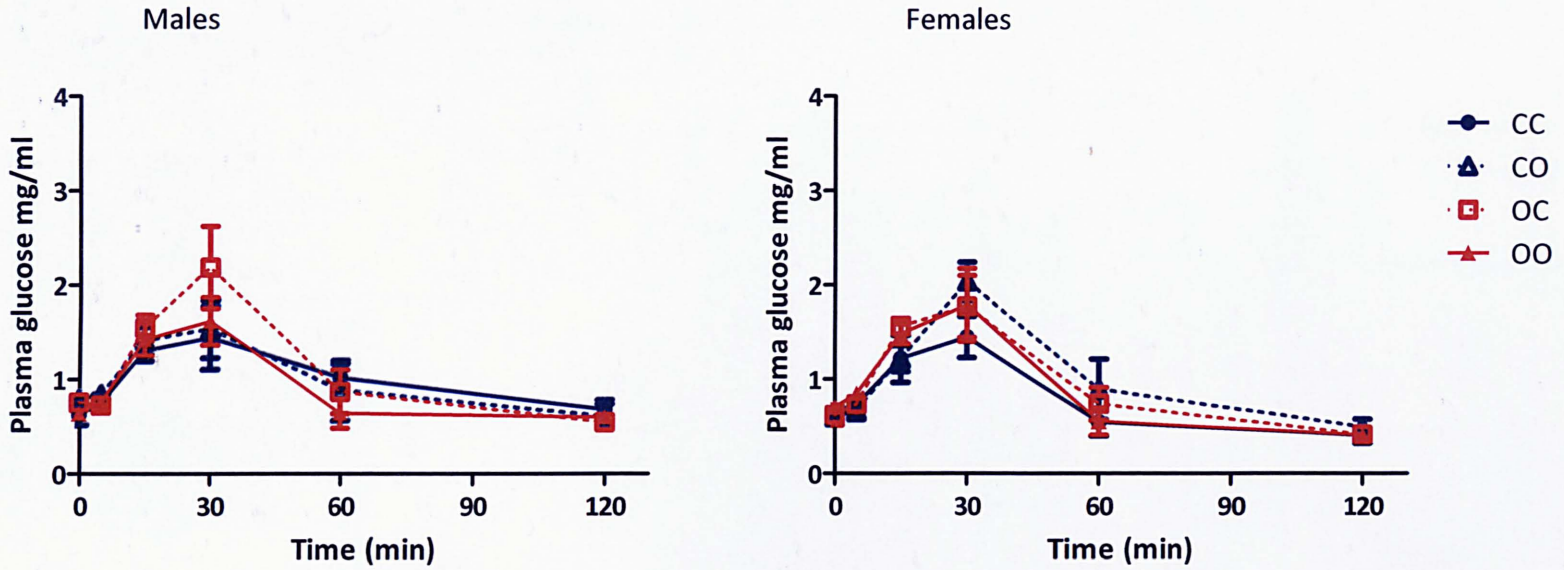


Figure 4.18 Plasma glucose concentrations following intraperitoneal glucose tolerance test of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning. Data was analysed at each time point separately for the effects of gender, pre-gestational and pregnancy diets. Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and n=6 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=5 for males and n=6 for females). All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. Gender significantly influenced glucose concentrations at the time points of baseline and 120 minutes ($P<0.05$).

Figure 4. 19 Expression of genes in the insulin signalling pathway of offspring exposed to cafeteria diet during lactation and chow diet during post-weaning

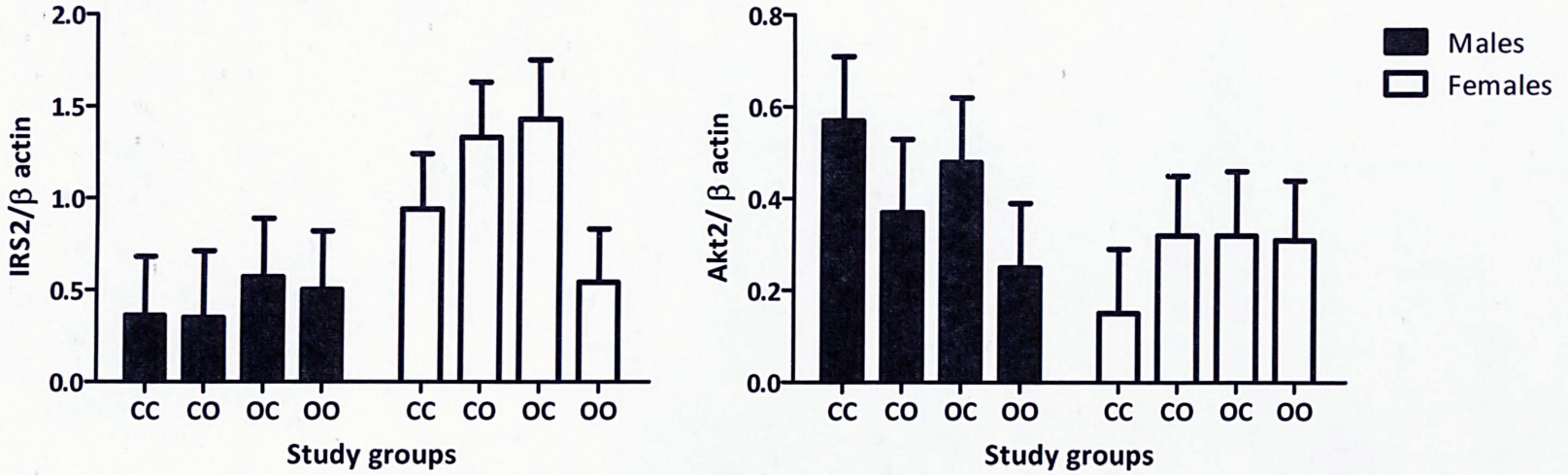


Figure 4.19 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to cafeteria diet during lactation and were weaned onto chow diet. Gender significantly influenced IRS2 mRNA expression ($P<0.05$).

4.4.4 Comparison of the Effects of Chow Diet versus Cafeteria Diet During Lactation on Offspring When the Post-Weaning Diet is Chow

Cafeteria diet during lactation induced some alterations in offspring metabolism but these alterations generally exhibited interactions with pre-gestational and pregnancy diets. When the weaning body weights were analyzed, interestingly an interaction between pregnancy and lactation diets showed that the offspring of XCO (X: C or O pre-gestational diet) and XOC (X: C or O pre-gestational diet) had significantly lower body weights (Male offspring: 65.28 ± 2.58 g in CCC versus 56.82 ± 2.29 in CCO, 54.47 ± 2.18 g in OCO, 54.30 ± 2.59 g in COC, 55.79 ± 2.66 g in OOC; female offspring: 61.15 ± 2.57 in CCC versus 56.23 ± 2.25 g in CCO, 53.56 ± 2.09 g in OCO, 52.02 ± 2.27 g in COC and 53.78 ± 2.68 g in OOC, $P<0.05$) (Tables 4.1 and 4.8). Thus the weight achieved at weaning in rats was lower if there was a switch between chow and cafeteria diet at the end pregnancy.

In addition to this, when the average body weight data all through the study was analyzed it appeared that the offspring where mothers consumed cafeteria diet during lactation, had significantly lower body weights than the offspring where mothers were fed only the chow diet during lactation (Male offspring: 252.79 ± 1.63 g in chow diet lactation groups versus 247.88 ± 1.56 g in cafeteria diet lactation groups; female offspring: 173.88 ± 1.45 g in chow diet lactation groups versus 169.02 ± 1.71 g in cafeteria diet lactation groups, $P<0.05$). Further interaction between pre-gestational, pregnancy and lactation diets indicated that the offspring of the group OCO had the lowest average

body weight throughout the study (Male offspring: 255.11 ± 3.13 g in CCC versus 228.67 ± 3.25 g in OCO; female offspring: 175.71 ± 3.36 g in CCC versus 156.97 ± 3.30 g in OCO, $P<0.05$).

Analysis of food intake showed that the female offspring of XCO (X: C or O pre-gestational diet) and XOC (X: C or O pre-gestational diet) had significantly increased energy intake when the data was expressed as kj consumed per kg of body weight (1488.00 ± 102.68 kj/day/kg in CCC versus 1662.06 ± 102.57 kj/day/kg in CCO, 1545.15 ± 101.6 kj/day/kg in OCO, 1672.64 ± 87.21 kj/day/kg in COC and 1722.94 ± 109.97 kj/day/kg in OOC, $P<0.05$) (Tables 4.6 and 4.10).

Plasma cholesterol concentrations were influenced by the dietary treatment during lactation. Offspring where mothers were fed by cafeteria diet during lactation had significantly higher plasma cholesterol concentrations than the offspring of the dams fed by chow diet during lactation (Male offspring: 1.78 ± 0.09 mmol/l in C during lactation versus 1.91 mmol/l in O during lactation; female offspring: 1.79 ± 0.09 mmol/l in C during lactation versus 2.09 ± 0.08 mmol/l in O during lactation, $P<0.05$).

Offspring of CXO (X: C or O pregnancy diet) and OXC (X: C or O pregnancy diet) had significantly lower baseline insulin concentrations than the control group (Male offspring: 0.61 ± 0.16 ng/ml in CCC versus 0.33 ± 0.09 ng/ml in CCO, 0.52 ± 0.03 ng/ml in COO, 0.49 ± 0.11 ng/ml in OCC, 0.32 ± 0.06 ng/ml in OOC; female offspring: 0.31 ± 0.07 ng/ml in CCC versus 0.30 ± 0.08 ng/ml in CCO, 0.07 ± 0.02 ng/ml in COO, 0.29 ± 0.06 ng/ml in OCC and 0.18 ±

0.05 ng/ml in OOC, P<0.05) (Tables 4.7 and 4.11). This suggested that the impact of cafeteria diet in lactation upon insulin secretion may be influenced by maternal adiposity (interaction of lactation and pre-gestational diet, P<0.05). Plasma insulin concentrations at 30 minutes post-intraperitoneal glucose injections were not influenced by lactation diet. An interaction between gender and lactation diet revealed that the area under curve glucose data was significantly lower in male offspring of the groups which were fed by cafeteria diet during gestation (141.10 ± 7.57 mg/ml.min in the groups that had chow diet during lactation versus 117.69 ± 6.56 mg/ml.min in the groups that had cafeteria diet during lactation, p<0.05). These results may suggest that insulin clearance in these groups was improved when compared to the control group. However, mRNA expression of IRS2 and AKT2 were not influenced by the dietary treatment during lactation.

4.4.5 Summary of the Findings

Table 4. 7 Summary of the findings when the offspring were exposed to chow diet during lactation and post-weaning

	Effects of Cafeteria Diet	
	Pre-gestation	**Pregnancy**
Fetal weight on day 20 of pregnancy	↓	-
Birth weight	↓	↑
Weaning weight	↔	↓
Post-weaning body weight	↓ (males)	↔
Gonadal fat pad	↔	↓ (females)
Peri-renal fat pad	↓ (males)	↔
Brain	↔	↓
Liver	Interaction ↓	
Left kidney	Interaction ↓	
Right kidney	↔	↔
Energy intake (kj/day)	↔	↔
Energy intake (kj/day/kg)	↔	↑(females)
Plasma cholesterol	↓	↔
Plasma triglyceride	↔	↔
Baseline glucose	↔	↔
Baseline insulin	↓	↔
30 min post glucose injection insulin	↓ (males)	Interaction with pre-gestation ↑
Δ insulin	↓ (males)	Interaction with pre-gestation ↑
AUC for glucose	↓ (females)	↔
mRNA expression of IRS2	↓ (males)	↔
mRNA expression of AKT2	↔	↔

Table 4.7 shows a brief summary of results from animals exposed to chow diet during lactation and post-weaning

Table 4. 8 Summary of the findings when the offspring were exposed to cafeteria diet during lactation and chow diet during post-weaning

	Effects of Cafeteria Diet	
	Pre-gestation	**Pregnancy**
Fetal weight on day 20 of pregnancy	↓	-
Birth weight	↓	↑
Weaning weight	↔	↑
Post-weaning body weight	↔	↑
Gonadal fat pad	↓	↔
Peri-renal fat pad	↓	↔
Brain	↔	↔
Liver	↔	↔
Left kidney	↔	↔
Right kidney	↔	↔
Energy intake (kj/day)	↑ (males)	↔
Energy intake (kj/day/kg)	↑ (males)	Interaction with pre-gestation ↑ (males)
Plasma cholesterol	Interaction ↓ (females)	
Plasma triglyceride	↔	↔
Baseline glucose	↔	↔
Baseline insulin	↔	↔
30 min post glucose injection insulin	↔	↔
Δ insulin	Interaction ↑	
AUC for glucose	↔	↔
mRNA expression of IRS2	↔	↔
mRNA expression of AKT2	↔	↔

Table 4.8 shows a brief summary of results from animals exposed to chow diet during lactation and post-weaning

4.5 Discussion

In this arm of the offspring trial, the individual and combined effects of maternal obesity and cafeteria diet feeding during pregnancy on body composition, appetite and glucose handling of the resulting offspring were assessed. Since dietary manipulations during the suckling period have been shown to be critically important in the development of adult-onset metabolic disorders both in humans and rodents *(Patel et al., 2009)*, an additional challenge was added to the study in order to determine the combined effects of over-nutrition during lactation and neo-natal life on the offspring's adult life. This created a comprehensive experiment but in order to determine the essential and causative time periods for programming effects, it was necessary to use an extremely complex study design. With this study design it was not feasible to analyze the body composition of the mothers, but in our maternal trials we clearly showed that cafeteria diet feeding for 8 weeks prior to pregnancy created a profound adiposity *(Akyol et al., 2009)*. As defined in Chapter 3, the study groups OCC and OCO that had chow diet only during pregnancy represented the individual effect of maternal obesity rather than the effect of diet in fetal/neonatal development. Groups COC and COO which had cafeteria diet only during pregnancy represented the effects of cafeteria diet during fetal development. Study groups OOO and OOC involved a combined effect of cafeteria feeding and maternal obesity.

Our results suggest that maternal cafeteria diet had some impact on the development and metabolism of offspring when fed at any stages of the

study (Tables 4.7 and 4.8). However, of these effects, maternal obesity was found to be the most influential factor on offspring growth and glucose homeostasis, since pre-gestational cafeteria diet exhibited not only independent effects but also modified responses to cafeteria diet during pregnancy and lactation (Tables 4.7 and 4.8). The difference between the effects of maternal obesity and high-fat diet during pregnancy on the resulting offspring are not clearly understood and only a few studies have examined this difference in the literature *(Shankar et al., 2008, White et al., 2008).* Despite using different experimental designs, these studies concluded that it was maternal obesity rather than the individual effect of high-fat diet during pregnancy which exerts the adverse programming effects on offspring. The influential effect of maternal obesity may be due to the greater impact of maternal reserves and substrate availability than high-fat diet during pregnancy. It is well known that substrate availability has profound effects on the metabolic interactions between the fetus, placenta and mother *(Simmons, 2007).* It was reported that in gestational diabetes increased maternal serum glucose and amino acids stimulate fetal pancreas to secrete more insulin and therefore the growth of fetal adipose tissue which may lead to macrosomia *(Simmons, 2008).* However, in the current study macrosomia was found to be related with high-fat feeding during pregnancy only and maternal metabolic biomarkers were not influenced grossly by maternal cafeteria diet *(Akyol et al., 2009).* Therefore, the decreased birth weight observed in offspring of obese mothers (pre-gestational cafeteria diet groups OC and OO) may be explained by intrauterine growth retardation or failure of

normal placental activities. It was shown in sheep and rat models that maternal obesity may lead to placental and growth retardation through decreased placental flow and stunted fetal growth *(Wallace et al., 2003, Milner and Hill., 1984).* Impaired placental synthesis of nitric oxide and placental synthesis of polyamines were considered as the responsible mechanisms of these outcomes *(Guoyao et al., 2004).* Placental nitric oxide acts as a major vasodilator and angiogenesis factor during pregnancy and therefore has important roles in regulating placental-fetal blood flow, transfer of nutrients and oxygen *(Bird et al., 2003).* Polyamines, which are the key regulators of DNA and protein synthesis, exert essential roles on cell proliferation and differentiation *(Flynn et al., 2002).* In Chapter 3 placental weight was shown to be influenced by cafeteria diet during pregnancy only which may indicate the growth restriction effects of maternal obesity (pre-gestational cafeteria diet) was not mediated by placental growth restriction. However, the link between maternal obesity, placental functions and decreased birth weight of pre-gestational cafeteria diet groups may suggest a further possibility. Therefore, assessment of placenta from these pregnancies is an important area for future study.

In parallel with our data, maternal high-fat feeding during pregnancy and lactation significantly increased the weaning body weight of offspring in other studies *(Howie et al., 2009, Tamashiro et al., 2009).* This increase in the body weights of offspring was confirmed to be due to greater adiposity *(Tamashiro et al., 2009).* However, decreased weaning body weights have

also been reported in the literature following a high-fat diet during pregnancy and lactation *(Cerf et al., 2005 and Ferezou-Viala et al., 2007).* In our study, we only observed this effect when the dietary treatment during lactation was chow diet and this effect was considered to be as a result of decreased energy intake of the cafeteria diet fed mothers during pregnancy which switched to chow diet during lactation.

Increased adiposity and hyperphagia due to the maternal high-fat diets or over-nutrition has been reported in several studies of rodents *(Shankar et al., 2008, Samuelsson et al., 2008, Bayol et al., 2008, Bayol et al., 2007, Buckley et al., 2005, Howie et al., 2009).* Within this context, our study exhibited unexpectedly contrasting results. Regardless of the dietary treatment during lactation, gonadal and peri-renal fat pad masses were lower in both pre-gestational or pregnancy cafeteria diet groups. When offspring were weaned onto chow diet during lactation and post-weaning, we observed consistent growth retardation since the birth weights, body weights, some organ sizes (% of body weight), fat pad masses (% of body weight) and baseline insulin levels were decreased with respect to other groups under the effects of pre-gestational cafeteria diet (Table 4.7 and 4.8). Similar phenotype with a reduction in body weight was associated with reduced glucose and insulin levels in offspring of the rats fed a low protein diet *(Arantes et al., 2002, Cherif et al., 2001).* Food intake of the groups did not show any marked difference when the dietary treatment during the suckling period was chow diet. On the other hand, when the dietary treatment during suckling period

was cafeteria diet male offspring of OCO and OOO groups exhibited 18.6 % and 8.6 % higher food intake. However, this did not lead these animals to increase their adiposity.

When these results are compared to those using similar study designs, for instance *Bayol et al, (2007)*, it becomes very difficult to explain the observed outcomes, as similar dietary protocols were used in both studies. However, in our trial a pre-gestational diet was introduced for 8 weeks before mating. Bayol *et al.,* initiated cafeteria feeding only at the start of pregnancy which may explain the differences between studies. Another difference between the current study and that of Bayol *et al.,* was that the Bayol group kept litters which contained between 10-16 pups, without reducing the litter size to a certain number at birth *(Bayol et al., 2007 and 2008).* In the present study, for each litter 8 offspring (4 males and 4 females) were kept. This may suggest another reason for the observed differences in results. It is clearly established that the rate of growth and development of rat pups during the suckling period is dependent on the litter size *(Chahoud and Paumgartten, 2009).* As discussed in Chapter 3, the range of the cafeteria diet foods was relatively small in our trial and resulted in an energy intake which was lower than reported in other studies of maternal obesity. Since hyperphagia is considered as one of the most important triggering factors in programming of obesity, the maternal exposure in the current trial may not have been enough to induce the previously observed effects.

In the literature most of the studies which have considered effects of maternal high-fat feeding and obesity reported some disturbance in glycaemic control of the offspring *(Tamashiro et al., 2009, Chen et al., 2008, Srinivasan et al., 2006, Buckley et al., 2005, White et al.,2009, Bayol et al.,2009, Shankar et al., 2008).* In the current trial, glucose homeostasis did not exhibit major disturbance associated with the maternal cafeteria diet. Interestingly, when the dietary treatment during lactation was chow diet, offspring of the rats that were fed by cafeteria diet during pre-gestational period only (OCC) showed an improved glucose control. Evidence of significantly lower insulin secretion, with a normal area under the glucose curve in males, and normal insulin levels with a lower area under the glucose curve data in females was shown. This may have indicated that these animals could tolerate intraperitoneal glucose injection better than controls due to greater insulin sensitivity. Similarly, when the dietary treatment during lactation was cafeteria diet, no effect of maternal diet was observed on the glucose homeostasis of offspring. The groups COO and OCO exhibited greater Δ insulin which may suggest that rat in these groups needed to produce more insulin in order to keep normal glucose concentrations. Therefore, in this arm of trial, evidence of decreased insulin concentrations and greater insulin sensitivity were noted. Maternal obesity (pre-gestational cafeteria diet) was shown to be more influential on the appearance of these effects than cafeteria diet during pregnancy only (Tables 4.7 and 4.8).

Rodents without diabetes were shown to adapt to increase β cell mass to meet the insulin demands *(Flier et al., 2001).* Previously it was reported that programming effects of maternal nutrition may be worsened by aging (Langley et al., 1994, Erhuma et al., 2007, Samuelsson et al., 2008). In our trial, glucose homeostasis measurements were performed when the offspring were 3 months old. Whole body insulin sensitivity showed similar results in 3 months old offspring from high-fat diet and control diet fed rat mothers in another study *(Buckley et al., 2005).* Therefore, assessing these offspring at an older age may elucidate more adverse effects of exposure to maternal cafeteria diet feeding.

In the current study, mRNA expression of IRS2 and AKT2 were measured in liver to assess whether glucose homeostasis was influenced by maternal cafeteria diet at the molecular level. In parallel with plasma glucose and insulin concentrations, no marked difference was found between the groups. When the litters were fed by chow diet during lactation, male offspring of OCC and OOC exhibited lower mRNA expression of IRS2 only. These data suggest that offspring of cafeteria diet fed animals exhibited no major difference in insulin signalling (Tables 4.7 and 4.8). However, other aspects of insulin signalling pathway may need to be considered. Shankar *et al.,* showed via immunoblotting that rats exposed to maternal high-fat diet during pregnancy increased their hepatic insulin receptor levels *(Shankar et al., 2010).* Bayol *et al.,* reported the upregulation of insulin receptor mRNA in male offspring which were exposed to cafeteria diet during post-weaning,

pregnancy and lactation or all through life whereas female offspring that were fed by cafeteria diet all through life exhibited lower expression levels *(Bayol et al., 2010).* In their study regulation of glucose transporters were also shown to be altered. Since gene expression analysis may give a limited picture when compared to protein synthesis analysis, examining related steps of insulin signalling mechanism at the protein level becomes crucial for future studies.

In conclusion, this chapter of our study showed that body weight, body composition, food intake or glucose homeostasis was not strongly influenced by exposure to maternal obesity and/or maternal cafeteria diet feeding in early life. Interestingly, when the offspring were weaned onto chow diet during the post-natal life, insulin sensitivity was observed to be improved. These outcomes were found to be more attributable to the maternal obesity rather than cafeteria diet during pregnancy alone. At the same time, cafeteria diet during lactation did not exert a major influence. Since an additional post-natal dietary challenge of continued high-fat diet during post-weaning period was shown to worsen the health status of offspring that were exposed to maternal over feeding when compared to a low fat diet during post-weaning period (*Khan et al., 2004*), exposure of the current groups to cafeteria diet post weaning was of great importance.

5.0 THE EFFECTS OF MATERNAL AND POSTNATAL CAFETERIA DIET ON GROWTH, DEVELOPMENT AND GLUCOSE HOMOEOSTASIS OF THE OFFSPRING

5.1 Introduction

As introduced in previous chapters, both epidemiological and experimental studies have demonstrated adverse effects of maternal obesity upon both maternal and fetal health status *(King, 2006).* It has been shown that feeding a cafeteria diet in rat pregnancy can induce altered food preferences and greater weight gain in the resulting offspring on rats *(Bayol et al., 2007).* In that study Bayol *et al.*, showed that when the chance of food preference (cafeteria diet during post-weaning) was given to offspring of cafeteria diet fed mothers during pregnancy and/or lactation, these offspring tended to consume more cafeteria diet foods than chow diet. The important effect of post-natal environment was demonstrated on human and animal studies by alterations in blood pressure, cholesterol and insulin levels (Singhal et al., 2001, Srinivasan et al., 2003, Beierle et al., 2004,). It was suggested that post-natal environmental factors may play an important role for assessing the adverse effects of peri-natal factors in critical period of growth *(Vuguin, 2007).*

According to the PAR hypothesis, fetus makes adaptations in utero based on the predicted post-natal envionment *(Gluckman and Hanson, 2004).* When this PAR is appropriate, the phenotype was suggested to be normal.

However, where a mismatch occurs between the predicted and actual environment, disease manifests. Khan *et al.,* reported that offspring of high-fat fed mothers were significantly heavier at 6 months of age when exposed to high-fat diet during post-natal life. This was associated with significant increase in gross energy intake, but no increase in adiposity *(Khan et al., 2004).* It was suggested that the results may indicate a PAR to the maternal high fat diet. Therefore, in the light of these suggestions, it was considered that exposing the offspring of cafeteria diet fed animals to a further dietary challenge in post-natal life may bring out a different phenotype in this study.

This chapter progresses Chapter 3 by investigating the effects of maternal cafeteria diet during pre-gestation and/or pregnancy on the growth and development and glucose homeostasis of offspring when the post-weaning nutritional treatment is cafeteria diet.

5.2 Objectives

This chapter of the study aimed to

i. Assess the effects of maternal cafeteria diet and/or maternal obesity on the growth and nutritional preferences of offspring until 3 months of age when the post-natal nutritional environment is cafeteria diet.
ii. Explore the effects of maternal cafeteria diet and/or maternal obesity on glucose homeostasis of offspring at 3 months of age when the post-natal nutritional environment is cafeteria diet.
iii. Compare the additional effects of chow or cafeteria diet during lactation on the previous parameters.

5.3 Materials and Methods

This chapter describes data from the Offspring Trial described in Section 2.1.4. It deals specifically with offspring that were weaned onto cafeteria diet. All methods are described in full in Chapter 2.

5.3.1 Biochemical Endpoints

Intraperitoneal glucose tolerance tests were performed when the animals were 13 weeks old (as described in 2.2.5). Fasting and post glucose injection plasma insulin levels were measured by commercially available Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem Inc, USA) as described in section 2.2.3.4. Plasma non-fasted glucose, cholesterol and triglyceride concentrations were measured through an adaptation of the glucose oxidase method and commercially available kits (as described in 2.2.3.1, 2.2.3.2 and 2.2.3.3). Whole body carcass analysis was performed using Soxhlet and Kjheldal methods (as described in 2.2.6). Expression of IRS2 and Akt2 were measured in liver as described in section 2.3.

5.3.2 Statistical Analyses

Data was analyzed using the Statistical Package for Social Sciences (version 18; SPSS, Inc., Chicago, IL, USA). Values are presented as mean ± SEM. The effects of maternal dietary treatment on offspring data were assessed using a mixed model ANOVA with fixed factors of pre-gestational diet, gestational diet and gender and random effect of litter size. Study weeks were added as a fixed factor where the nutritional data was analyzed. Body weights and energy intakes were assessed by repeated

ANOVA. P<0.05 was considered as significant unless otherwise indicated. No post hoc tests were performed. Symbols used in tables and figures represent main effects indicated by ANOVA.

5.4 Results

5.4.1 Reproductive Outcome

All of the animals mated normally. Reproductive outcome and birth weight data was presented in section 4.4.1.

5.4.2 Chow diet during lactation and cafeteria diet during post-weaning period

5.4.2.1 Growth and body composition

Body weight during lactation was presented in Section 4.4.2.1, Figure 4.2. When the post-weaning dietary treatment was cafeteria diet, animals continued to follow the pattern that was observed during lactation. Cafeteria diet in pregnancy induced a tendency for offspring to have lower body weights in both genders (P=0.053) (Figure 5.1). In addition to this, significant interactions between pre-gestational diet and gender, and pre-gestational diet, pregnancy diet and gender indicated that male offspring exposed to cafeteria diet at any stage of the study had lower body weights than controls (Final week body weights: 483.50 ± 20.26 g in CC versus 429.10 ± 18.12 g in CO, 448.12 ± 16.54 g in OC and 427.13 ± 20.26 in OO, P<0.05). Gender and age significantly influenced weight (P<0.05). These animals were significantly heavier than the corresponding chow diet post-weaned animals (P<0.05).

When the animals were 13 weeks old gonadal and peri-renal fat pads and organ weights (percentage of body weight) were not significantly influenced by pre-gestational or pregnancy diets ($P>0.05$) (Table 5.1). Whole body carcass analysis results were similar between the groups ($P>0.05$) (Table 5.1). Females had heavier gonadal fat pad and brain mass than males whereas males had heavier peri-renal fat pad and liver mass than females ($P<0.05$) (Table 5.1).

Figure 5. 1 Average body weights of male and female offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

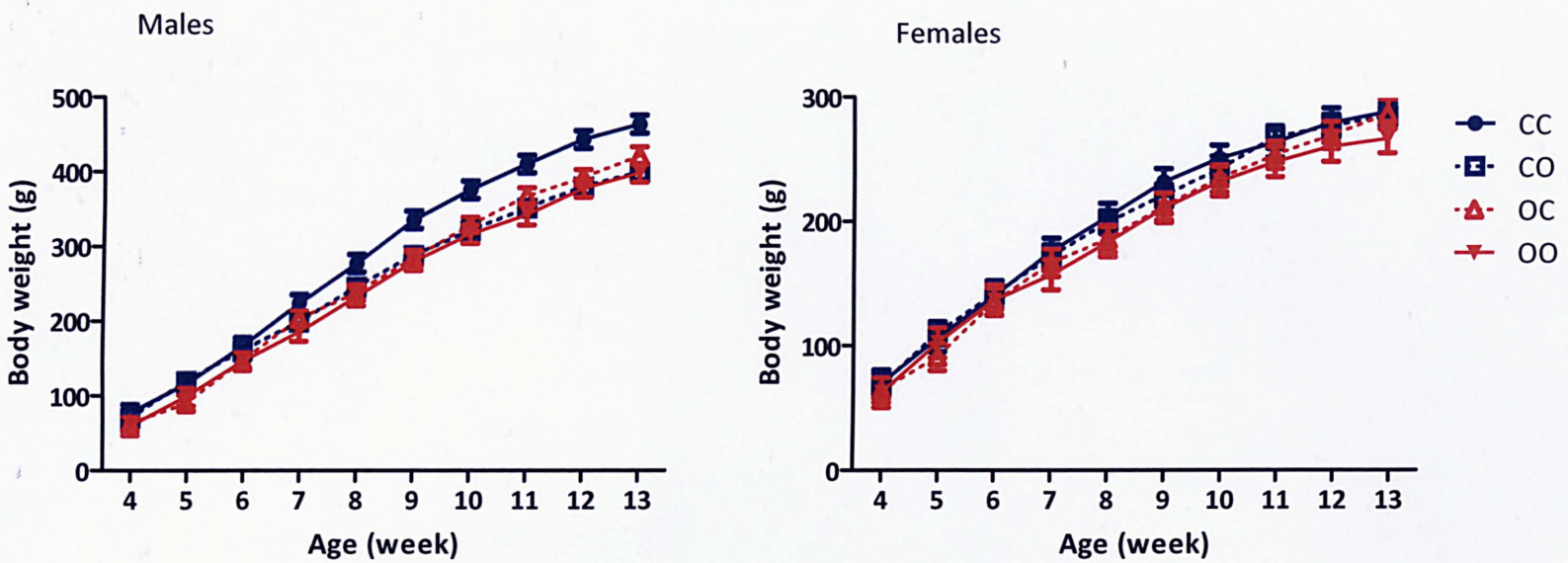


Figure 5.1 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and were weaned onto cafeteria diet. There was a tendency for the gestational cafeteria diet groups to have lower body weights (P=0.053). The effect of gender and study weeks were significant (P<0.001).

Table 5. 1 Body composition of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

		CC (n=4 or 5)		CO (n=5 or 6)		OC (n=6)		OO (n=4)	
Sex	% of body weight	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Gonadal fat	4.28‡	0.92	3.49‡	0.36	3.68‡	0.28	3.95‡	0.30
	Peri-renal fat	4.25‡	0.55	3.53‡	0.33	3.44‡	0.29	3.78‡	0.28
	Brain	0.40‡	0.01	0.44‡	0.01	0.41‡	0.01	0.42‡	0.02
	Liver	2.86‡	0.03	2.69‡	0.09	2.78‡	0.10	2.69‡	0.11
	Right kidney	0.27	0.01	0.28	0.01	0.27	0.02	0.29	0.02
	Left kidney	0.26	0.009	0.28	0.02	0.27	0.02	0.26	0.02
	Total nitrogen	7.75	0.23	7.98	0.24	6.99	0.24	7.32	0.23
	Total fat	48.87	1.64	49.10	1.54	48.56	1.64	48.83	1.63
	Body water	44.68	2.05	45.6	2.18	45.2	2.03	44.9	2.05
Female	Gonadal fat	6.18‡	0.90	5.74‡	0.51	5.84‡	0.27	5.88‡	0.44
	Peri-renal fat	3.30‡	0.17	3.35‡	0.26	3.18‡	0.25	3.47‡	0.30
	Brain	0.59‡	0.05	0.59‡	0.02	0.57‡	0.02	0.57‡	0.05
	Liver	2.41‡	0.07	2.47‡	0.11	2.46‡	0.06	2.40‡	0.07
	Right kidney	0.27	0.02	0.26	0.01	0.26	0.004	0.27	0.008
	Left kidney	0.26	0.01	0.25	0.01	0.25	0.01	0.26	0.01

Table 5.1 Body composition of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning (continued)

		CC (n=4 or 5)		CO (n=5 or 6)		OC (n=6)		OO (n=4)	
Sex	% of body weight	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Female	Total nitrogen	7.05	0.19	6.98	0.23	6.99	0.23	7.00	0.23
	Total fat	46.98	1.41	47.54	1.35	46.54	1.30	47.98	1.31
	Body water	49.96	1.43	48.65	1.38	49.10	1.38	48.85	1.39

Table 5.1 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. ‡ indicates the significant effect of gender ($P<0.001$).

5.4.2.2 Nutritional intakes

The average amount of energy consumed per day across the study was significantly lower in pre-gestational cafeteria diet groups (3.5 % lower in OC and 5.8 % in OO with respect to CC) than pre-gestational control groups in males ($P<0.05$) (Table 5.2 A and B, Figure 5.2). Other components of the diet did not show any significant differences when the data was expressed as the daily intakes (Table 5.2 A and B, Figure 5.3, 5.4 and 5.5). However, when the data was expressed as the energy consumed per kg of body weight, there were significant effects of pregnancy diet and the interaction between pre-gestational and pregnancy diets in both genders ($P<0.05$) (Table 5.2 A and B). All of the groups exposed to cafeteria diet during pre-gestation or pregnancy (CO, OC and OO) consumed significantly more energy than CC during the post-weaning period (male offspring: 7.6 % more in CO, 14.9 % in OC and 10.4 % in OO, female offspring: 6.1 % more in CO, 12.2 % in OC and 11.0 % in OO with respect to CC) (Table 5.2 A and B). This situation was also noted for intakes of micronutrients such as fat (male offspring: 10.6 % more in CO, 18.6 % in OC and 11.7 % in OO, female offspring: 3.5 % more in CO, 7.4 % in OC and 9.2 % in OO with respect to CC), protein (male offspring: 2.3 % more in CO, 8.2 % in OC and 3.4 % in OO, female offspring: 11.6 % more in CO, 14.2 % in OC and 13.0 % in OO with respect to CC), carbohydrate (male offspring: 9.8 % more in CO, 13.2 % in OC and 6.0 % in OO, female offspring: 12.6 % more in CO, 19.4 % in OC and 17.1 % in OO with respect to CC) and

saturated fatty acids (male offspring: 9.8 % more in CO, 13.2 % in OC and 6.0 % in OO, female offspring: 12.6 % more in CO, 19.4 % in OC and 17.1 % in OO with respect to CC) (Table 5.2 A and B). Sugar intake per kg of body weight was significantly higher in offspring of pregnancy cafeteria diet animals than control animals (male offspring: 10.8 % more in CO, 32.8 % more in OO, female offspring: 10.9 % more in CO, 18.2 % more in OO). Sodium intake per kg of body weight was significantly higher in the groups CO and OC (significant interaction between pre-gestational and gestational diets, $P<0.05$) (male offspring: 34.0 % more in CO, 19.1 % more in OC, female offspring: 17.4 % more in CO, 19.6 % more in OC). Gender and age significantly influenced the nutrient intakes of the animals ($P<0.001$). Average energy intake of the groups were significantly higher than the corresponding chow diet post-weaned groups ($P<0.05$).

When the amount of chow diet consumed throughout the study was analyzed, an interesting interaction between pre-gestational diet, pregnancy diet and gender showed that the female offspring of the groups CO and OC consumed more chow than the groups CC and OO ($P<0.05$) (Figure 5.6). Male offspring did not exhibit this effect.

5.4.2.3 Circulating lipids

The effects of maternal cafeteria diet on plasma cholesterol and triglyceride concentrations are shown in Figure 5.7. Interestingly, animals

exposed to cafeteria diet in pregnancy exhibited a trend towards lower plasma triglyceride concentrations (P=0.05) (Figure 5.7), whereas plasma cholesterol concentrations were similar in all groups (Figure 5.7). When compared to chow diet post-weaned animals, cafeteria diet post-weaned animals had similar plasma cholesterol concentrations (P>0.05) whereas plasma triglyceride concentrations were significantly higher (P<0.05).

Table 5. 2 Average nutrient intakes of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

		Post-weaning period							
		CC (n=4 or 5)		CO (n=5 or 6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Males	Energy intake								
	(KJ/day/kg)	1614.95	134.07	1737.87†¥	125.20	1855.79¥	109.67	1728.39†	140.19
	(KJ/day)	355.19‡	16.46	370.85‡	14.41	342.59‡*	15.22	334.69‡*	16.22
	Fat intake								
	(g/day/kg)	21.22	1.69	23.47†¥	1.61	25.17¥	1.57	23.59†	1.80
	(g/day)	4.91‡	0.21	4.98‡	0.18	4.87‡	0.19	4.69‡	0.21
	Protein intake								
	(g/day/kg)	14.19	1.21	14.52†¥	1.06	15.20¥	1.02	14.67†	1.17
	(g/day)	2.94‡	0.13	3.07‡	0.10	2.81‡	0.21	2.88‡	0.14
	CHO intake								
	(g/day/kg)	34.46	3.47	37.82†¥	3.43	39.02†¥	2.73	36.53	3.56
	(g/day)	7.25‡	0.59	8.02‡	0.53	6.94‡	0.66	6.68‡	0.91
	SFA intake								
	(g/day/kg)	34.46	3.86	37.82†¥	3.31	39.02¥	3.43	36.53†	3.81
	(g/day)	1.95‡	0.12	2.05‡	0.11	1.78‡	0.31	1.92‡	0.19

Table 5.2 (A) Average nutrient intakes of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning *(continued)*

		Post-weaning period							
		CC (n=4 or 5)		CO (n=5 or 6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Males	Sugar intake								
	(g/day/kg)	10.68	1.53	11.83†	1.32	11.75	1.36	14.18†	1.51
	(g/day)	2.34‡	0.24	2.46‡	0.19	2.30‡	0.21	2.64‡	0.23
	Na intake								
	(g/day/kg)	0.47	0.07	0.63¥	0.06	0.56¥	0.06	0.48	0.07
	(g/day)	0.11‡	0.001	0.13‡	0.001	0.11‡	0.001	0.09‡	0.003

Table 5.2 (A) Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. SFA: Saturated fatty acid. Na: Sodium. All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. * indicates the significant effect of pre-gestational cafeteria diet when compared to pre-gestational control animals ($P<0.05$). † indicates the significant effect of pregnancy cafeteria diet when compared to pregnancy control animals ($P<0.05$). ¥ indicates the significant interaction between pre-gestational and gestational diets ($P<0.05$). ‡ indicates the significant effect of gender difference on nutrient intakes ($P<0.001$).

Table 5.2(B) Average nutrient intakes of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

		Post-weaning period							
		CC (n=4 or 5)		CO (n=5 or 6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Females	Energy intake								
	(KJ/day/kg)	1735.18	119.40	1840.69†¥	123.61	1947.51¥	104.15	1926.27†	159.97
	(KJ/day)	290.04‡	15.35	322.97‡	13.87	293.70‡	15.15	293.24‡	16.35
	Fat intake								
	(g/day/kg)	23.11	1.54	23.91¥	1.61	24.83¥	1.44	25.23†	2.02
	(g/day)	4.08‡	0.19	4.09‡	0.17	3.93‡	0.16	3.88‡	0.32
	Protein intake								
	(g/day/kg)	13.77	1.01	15.37†¥	1.16	15.72¥	0.90	15.56†	1.29
	(g/day)	2.22‡	0.12	2.62‡	0.11	2.39‡	0.12	2.38‡	0.13
	CHO intake								
	(g/day/kg)	37.49	3.11	42.22†¥	3.51	44.75¥	2.71	43.91†	4.41
	(g/day)	5.91‡	0.55	7.59‡	0.51	6.49‡	0.55	6.53‡	0.58
	SFA intake								
	(g/day/kg)	37.49	3.54	42.22†¥	3.12	44.75¥	3.40	43.91†	3.56
	(g/day)	1.61‡	0.11	1.58‡	0.10	1.43‡	0.11	1.51‡	0.12

Table 5.2 (B) Average nutrient intakes of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning (continued)

		Post-weaning period							
		CC (n=4 or 5)		CO (n=5 or 6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Female	Sugar intake								
	(g/day/kg)	14.37	1.41	15.93†	1.25	14.56	1.35	16.98†	1.59
	(g/day)	2.49‡	0.21	2.54‡	0.25	2.66‡	0.32	2.69‡	0.17
	Na intake								
	(g/day/kg)	0.46	0.06	0.54¥	0.05	0.55¥	0.06	0.52	0.08
	(g/day)	0.08‡	0.002	0.09‡	0.001	0.08‡	0.001	0.08‡	0.001

Table 5.2(B) Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. SFA: Saturated fatty acid. Na: Sodium. All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. * indicates the significant effect of pre-gestational cafeteria diet when pregnancy control animals ($P<0.05$). ¥ indicates the significant interaction between pre-gestational and gestational diets ($P<0.05$). ‡ indicates the significant effect of gender difference on nutrient intakes ($P<0.001$).

Figure 5. 2 Average energy intakes of male and female offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

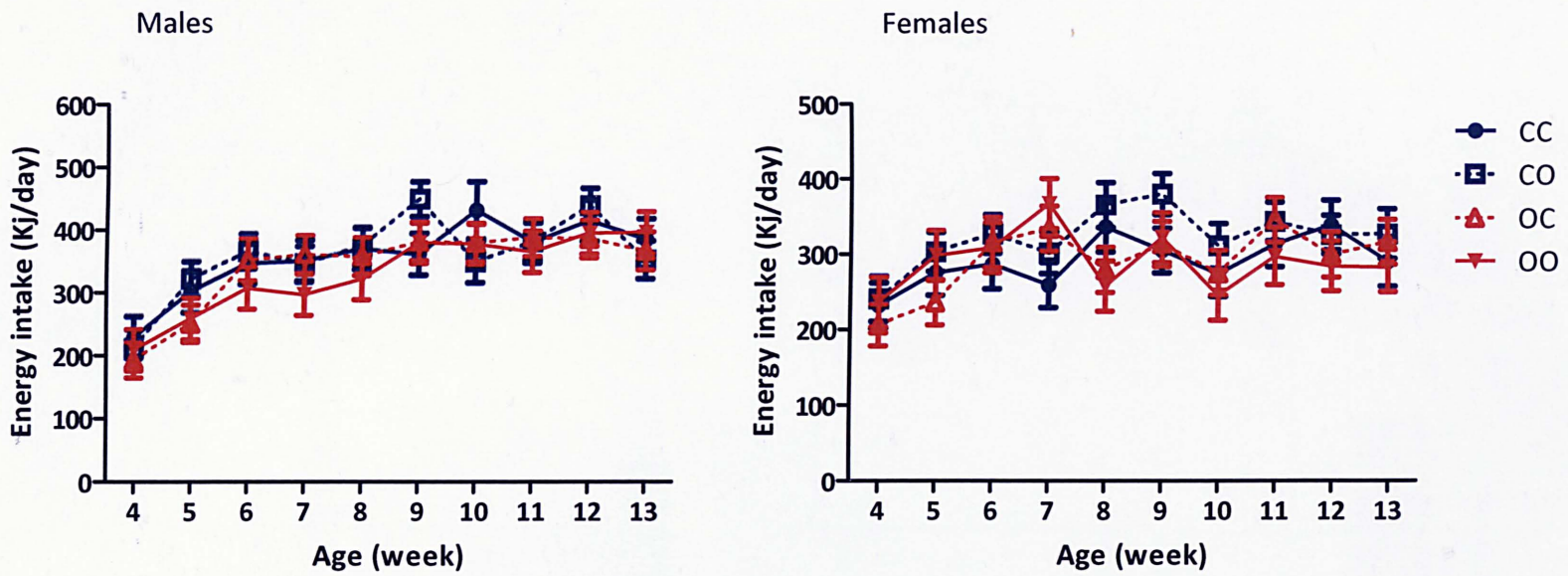


Figure 5.2 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. Repeated measures ANOVA showed that pre-gestational cafeteria diet groups had significantly lower energy intake ($P<0.05$). The effects of gender and age were significant ($P<0.001$).

Figure 5. 3 Average protein intakes of male and female offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

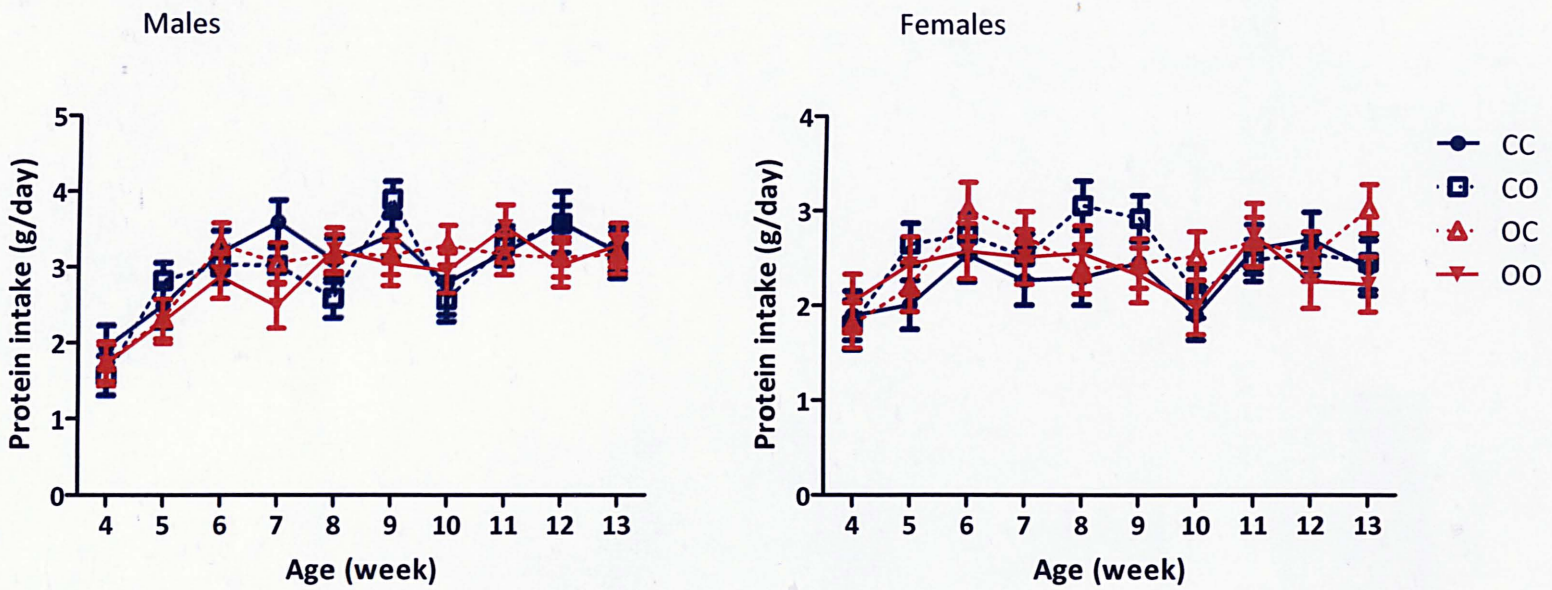


Figure 5.3 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. The effects of gender and age were significant (P<0.001).

Figure 5. 4 Average fat intakes of male and female offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

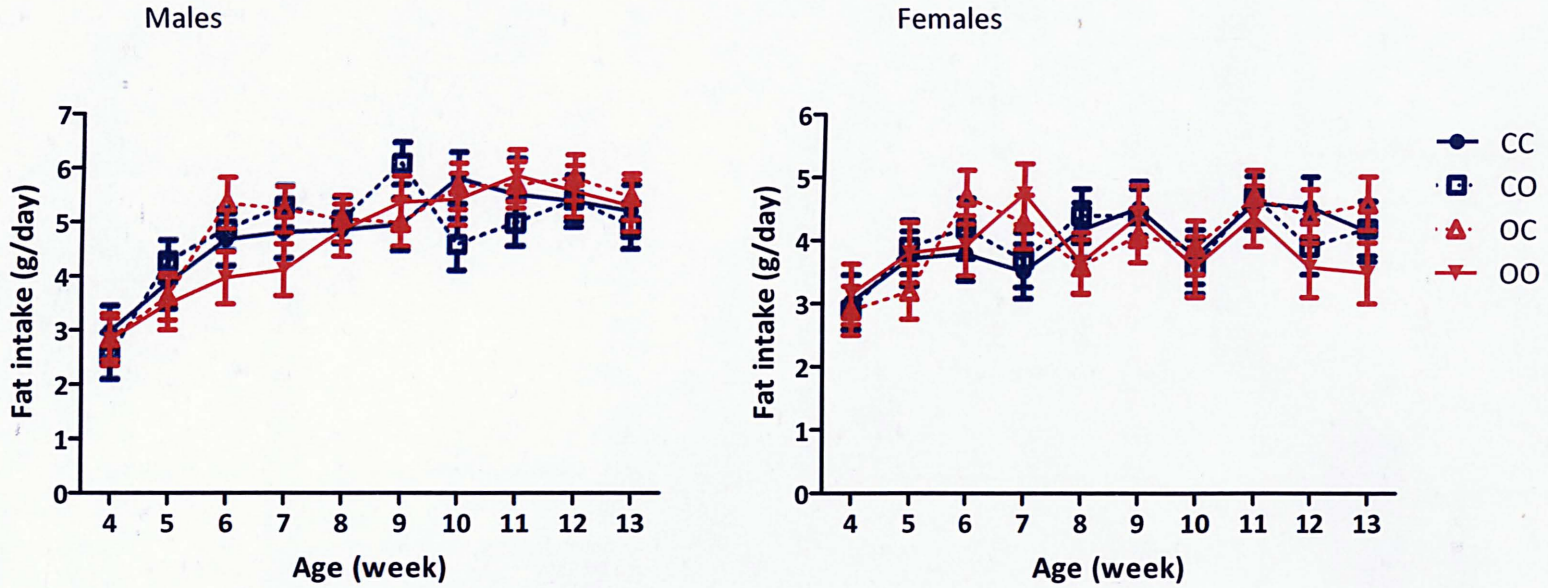


Figure 5.4 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. The effects of gender and age were significant ($P<0.001$).

Figure 5. 5 Average carbohydrate intakes of male and female offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

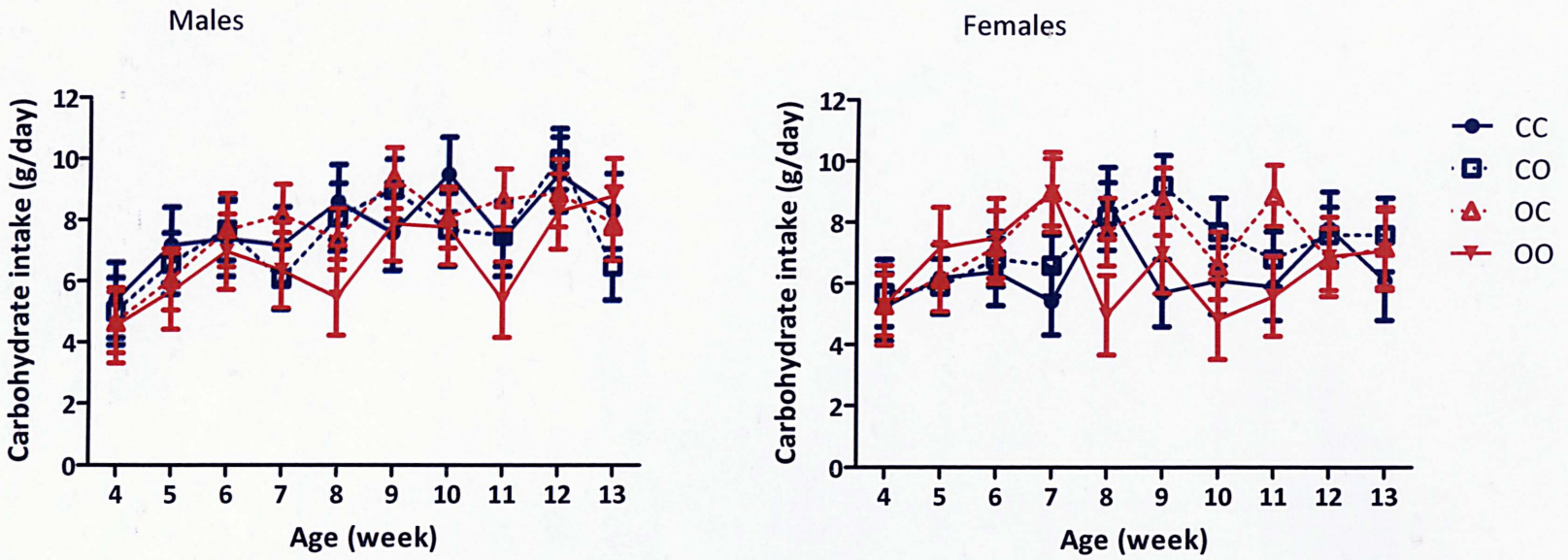


Figure 5.5 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. The effect of gender and age were significant (P<0.001).

Figure 5. 6 Average percentage of chow diet energy in male and female offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

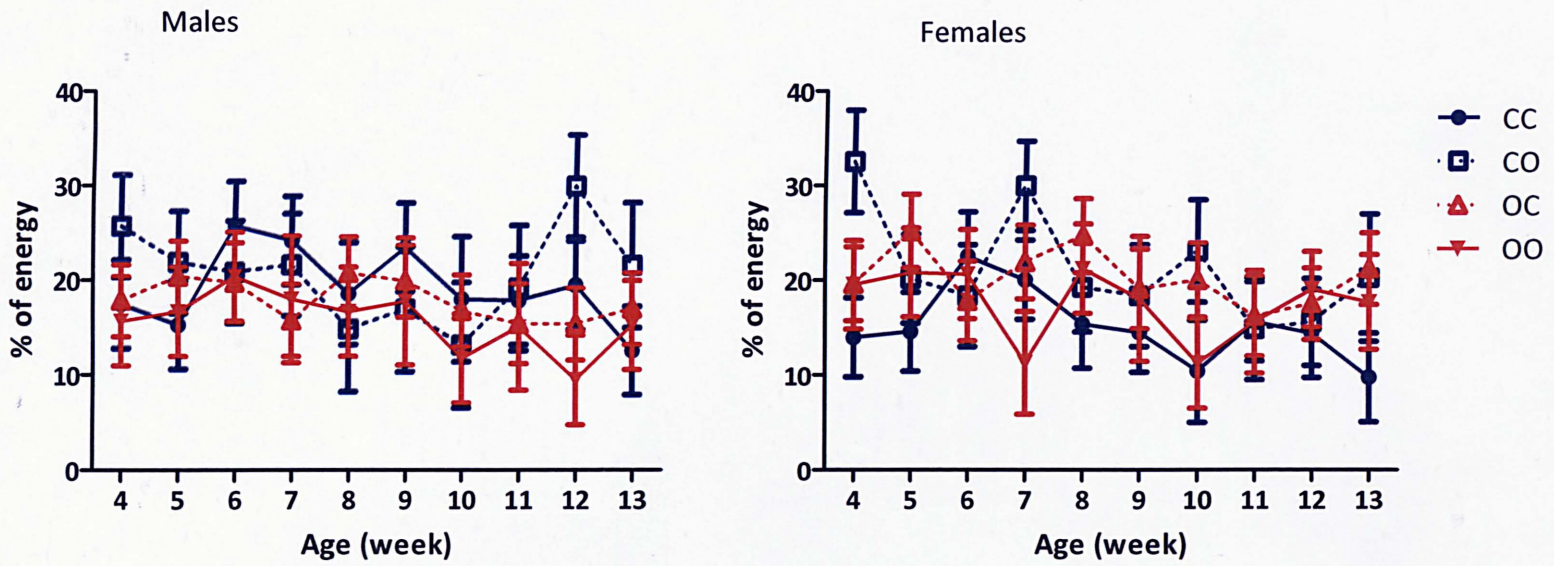


Figure 5.6 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. Offspring of CO and OC consumed significantly higher amounts of chow diet ($P<0.05$).

Figure 5. 7 Plasma cholesterol and triglycerides of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

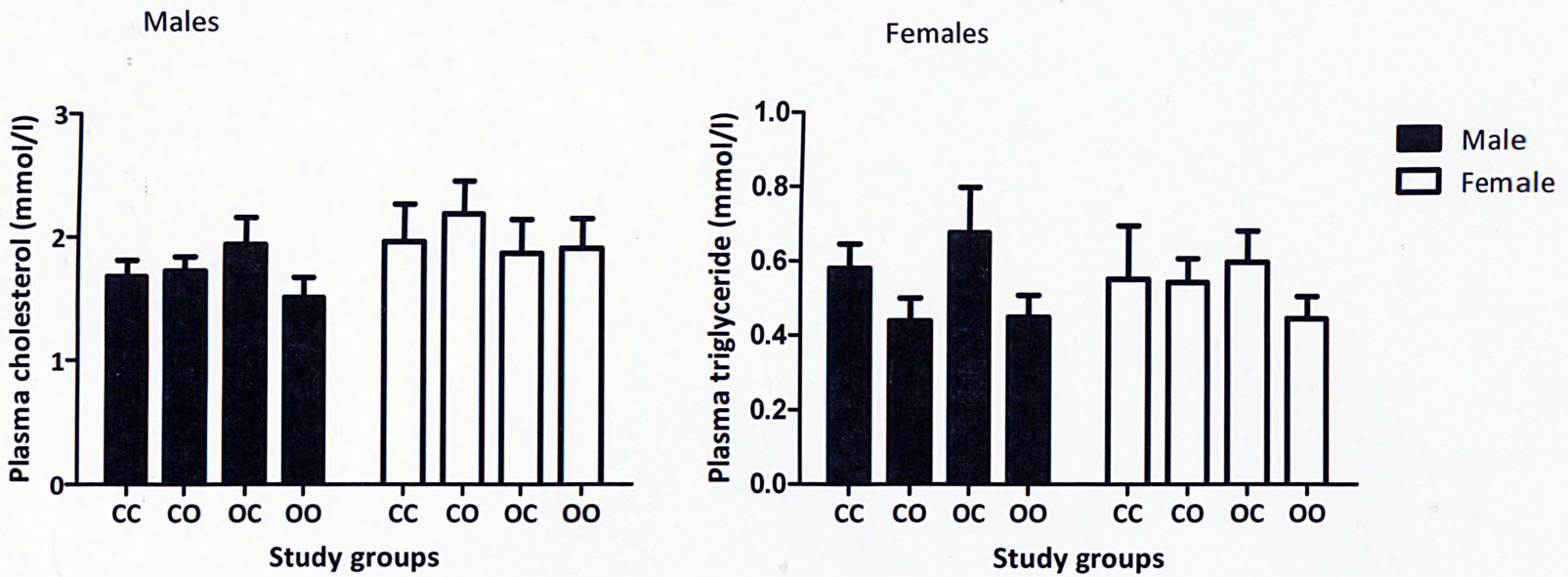


Figure 5.7 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and n=5 for females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=5 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females).All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. Gender significantly influenced plasma triglyceride concentrations ($P<0.05$).

5.4.2.4 Glucose homeostasis

When the dietary treatment was chow during lactation followed by cafeteria diet for the post-weaning period, there were important alterations in glucose homeostasis among offspring that were prenatally exposed to cafeteria diet. Although the baseline fasting glucose concentrations did not differ between the groups, male offspring of the rats fed cafeteria diet during pregnancy had significantly lower baseline fasting insulin concentrations (48.2 % lower in CO and 61.8 % lower in OO with respect to CC, $P<0.05$) (Table 5.3). Half an hour after the glucose injection, male offspring of the CO and OC groups exhibited a trend for higher insulin concentrations when compared to controls whereas this was only observed in the females of the OC group ($P=0.06$) (Table 5.3). When the Δ insulin (difference between baseline and thirty minute insulin concentrations) was assessed, female offspring of the pre-gestational cafeteria diet groups had significantly higher insulin responses to glucose administration than the pre-gestational control groups (43.1 % higher in OC and % 27.1 in OO with respect to CC) (interaction between pre-gestational diet and gender, $P<0.05$) (Table 5.4).

Fifteen minutes post glucose injection, glucose concentrations were significantly higher in the groups CO (2.16 ± 0.26 mg/ml in males and 1.92 ± 0.24 mg/ml in females) and OC (1.92 ± 0.24 mg/ml in males and 1.68 ± 0.27 in females) than CC (1.00 ± 0.29 mg/ml in males and 1.36 ± 0.23 mg/ml in females) (significant interaction between pre-gestational and gestational diets, $P<0.05$) and this effect lasted until two hours post-injection (tendency

towards an interaction between pre-gestational and pregnancy diets, P=0.08) (Figure 5.8). The area under the glucose curve data confirmed that these groups of offspring were exhibiting glucose intolerance. A significant interaction between pre-gestational and pregnancy diets showed that offspring of CO, OC and OO had significantly higher areas under the glucose curve data (73.3 % increase in CO, 77.1 % in OC and 41.5 % in OO in males, 15.2 % increase in CO, 35.9 % in OC and 17.5 % in OO in females with respect to CC, $P<0.05$, Table 5.3). This effect was particularly marked in female offspring of OC.

Analysis of IRS2 and AKT2 mRNA expression revealed that the observed glucose intolerance could be attributable to alterations of the insulin signalling pathway. Consistently, male offspring of CO and OC and female offspring of OC groups had significantly higher IRS2 mRNA expression in liver (significant interaction between pre-gestational and gestational diets, $P<0.05$) (Figure 5.9). There were no significant effects of maternal diet upon expression of AKT2 in the liver. Males of the CO and OC groups tended to have higher AKT2 mRNA expression, although this effect failed to achieve statistical significance (P=0.08 for interaction of gender, pre-gestational and pregnancy diets) (Figure 5.9).

Table 5. 3 Glucose tolerance data of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning period

		CC (n=4 or 5)		CO (n=5 or Fig6)		OC (n=6)		OO (n=4)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Baseline glucose (mg/ml)	0.80‡	0.08	0.82‡	0.07	0.78‡	0.07	0.72‡	0.08
	Baseline insulin (ng/ml)	1.99‡	0.33	1.03‡†	0.33	1.89‡	0.27	0.76‡†	0.33
	Insulin at 30 min (ng/ml)	2.15	0.83	3.64	0.83	3.49	0.68	1.66	0.83
	Δ insulin (ng/ml)	1.74	0.94	3.03	0.75	1.41	0.63	2.15	0.83
	AUC glucose (mg/ml.min)	95.42	23.72	165.38¥	21.22	169.01¥	19.37	135.05¥	19.76
Female	Baseline glucose (mg/ml)	0.72‡	0.07	0.62‡	0.07	0.63‡	0.08	0.60‡	0.05
	Baseline insulin (ng/ml)	0.31‡	0.29	0.31‡	0.29	0.29‡	0.27	0.18‡	0.16
	Insulin at 30 min (ng/ml)	2.60	0.74	2.26	0.68	4.24	0.74	3.32	0.92
	Δ insulin (ng/ml)	2.55	0.67	2.06	0.66	3.65∫	0.68	3.24∫	0.73
	AUC glucose (mg/ml.min)	126.60	21.22	145.44¥	19.37	172.12¥	21.22	148.18¥	23.72

Table 4.7 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All offspring were exposed to chow diet during lactation and weaned onto cafeteria diet. Δ Insulin: the difference between baseline and thirty minute insulin concentrations. † indicates the significant interaction between gestational diet and gender ($P<0.05$). ‡ indicates the significant effect of gender difference ($P<0.001$). ¥ indicates the significant interaction between pre-gestational and gestational diets ($P<0.05$). ∫ indicates the significant interaction between pre-gestational diet and gender ($P<0.05$).

Figure 5. 8 Plasma glucose concentrations following intraperitoneal glucose tolerance test of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

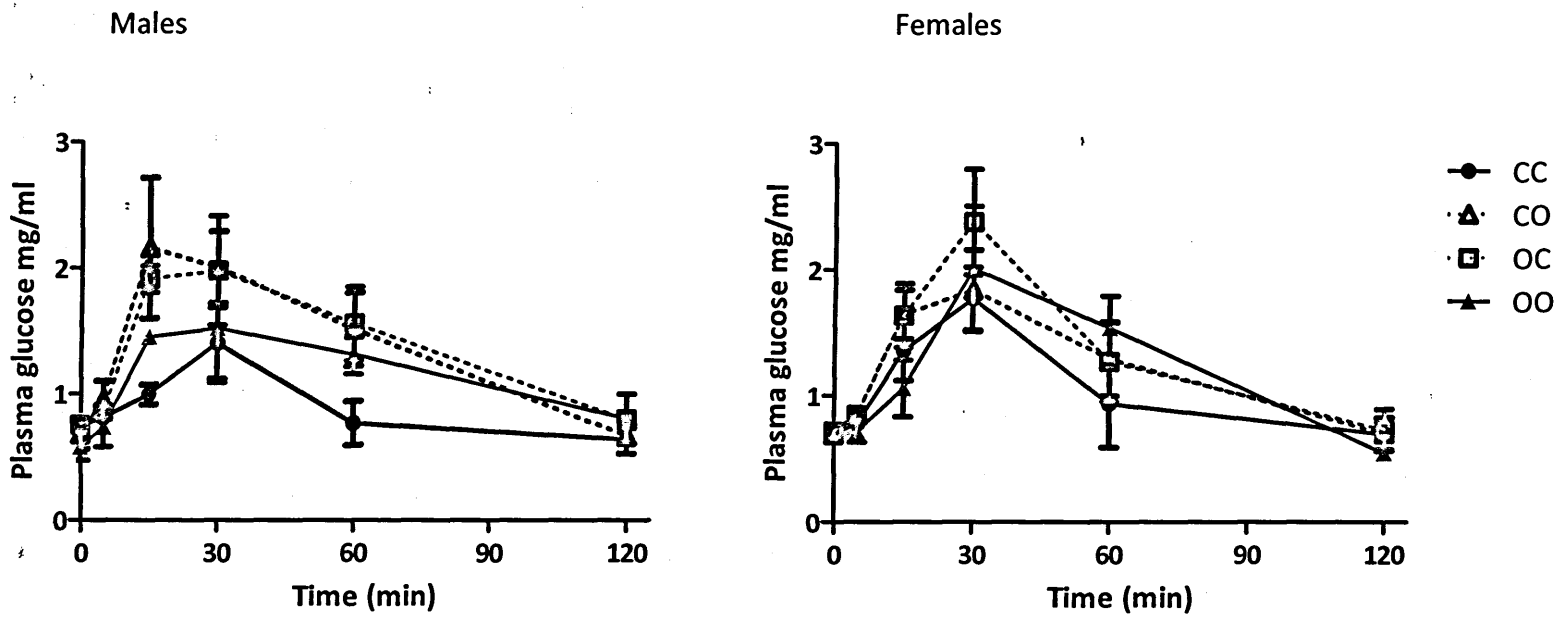


Figure 5.8 Data is shown as mean±SEM. Data was analysed at each time point separately for the effects of gender, pre-gestational and pregnancy diets. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. ¥ indicates the significant interaction between pre-gestational and gestational diets ($P<0.05$).

Figure 5. 9 Expression of genes in the insulin signalling pathway of offspring exposed to chow diet during lactation and cafeteria diet during post-weaning

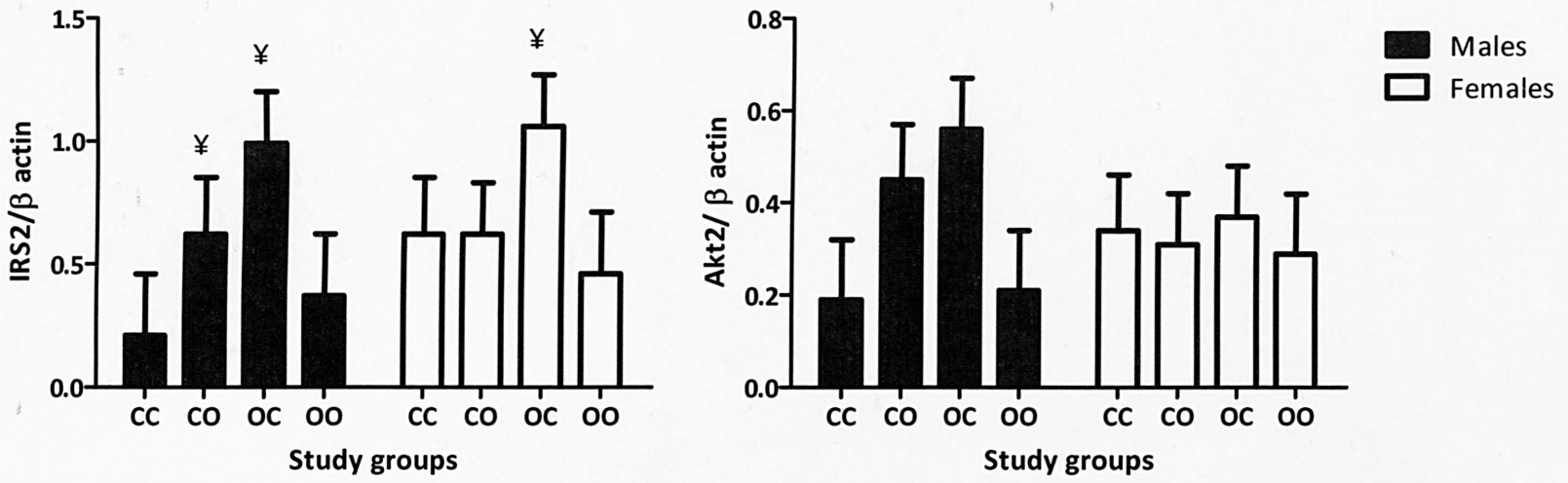


Figure 5.9 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=5 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=6 for males and females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=6 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and females). All groups were exposed to chow diet during lactation and weaned onto cafeteria diet. ¥ indicates the significant interaction between pre-gestational and gestational diets ($P<0.05$).

5.4.3 Cafeteria diet during lactation and post-weaning period

5.4.3.1 Growth and body composition

Body weight during lactation was presented in Section 4.4.2.1, Figure 4.2. When the offspring were weaned onto cafeteria diet after the same diet during lactation, the effects of the pregnancy cafeteria diet on body weights of the offspring up to weaning disappeared. Repeated measures ANOVA results showed that the body weights of these animals did not differ in the post weaning period ($P>0.05$) (Figure 5.10). Body weights of the animals increased with age and males were significantly heavier than the females ($P<0.001$) (Figure 5.10).

In parallel with the body weight data, body composition and organ sizes did not differ between the groups ($P>0.05$) (Table 5.4). Gonadal fat pad, peri-renal fat pad, liver and brain weights were significantly different between males and females ($P<0.05$) (Table 5.4). Male offspring had heavier peri-renal fat pads and liver whereas female offspring had heavier gonadal fat pads and greater brain mass relative to body weight (Table 5.4). This effect was probably due to the heavier body weights of male offspring.

Figure 5. 10 Average body weights of male and female offspring exposed to cafeteria diet during lactation and post-weaning

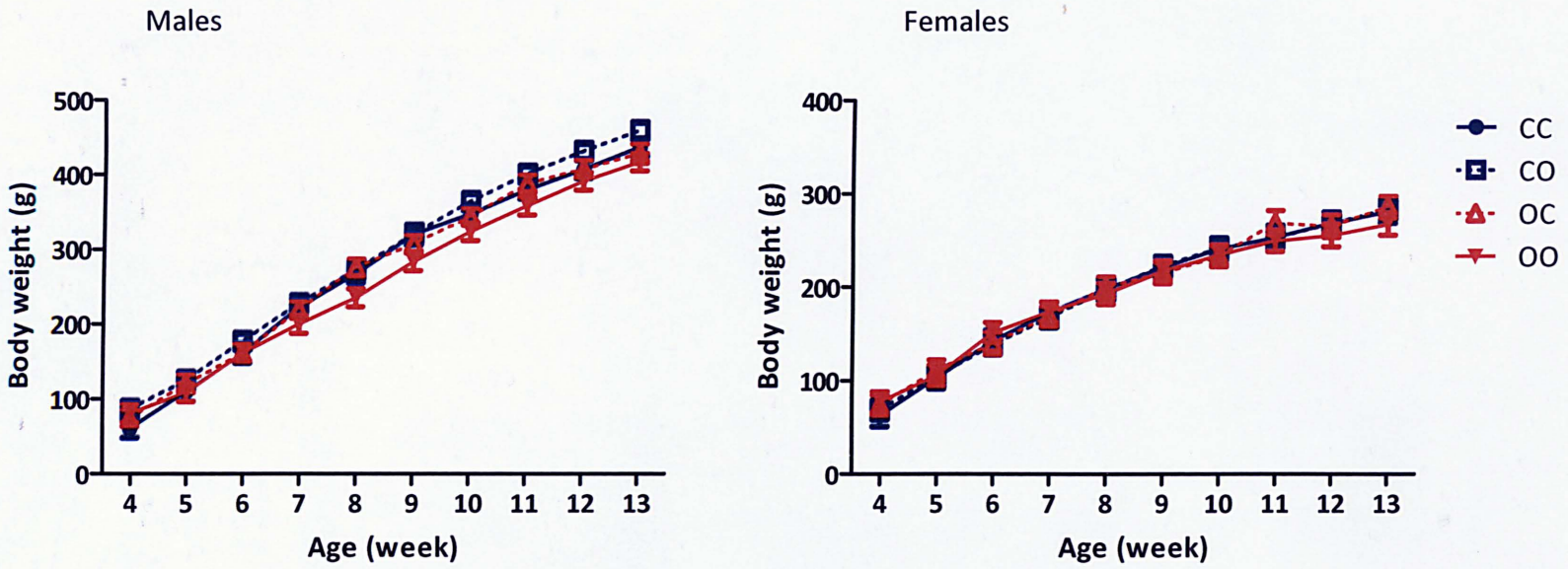


Figure 5.10 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. Gender and age significantly influenced body weights of the animals ($P<0.001$).

Table 5. 4 Body composition of offspring exposed to cafeteria diet during lactation and post-weaning

		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex	% of body weight	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Gonadal fat	4.20 ‡	0.71	5.05 ‡	0.71	4.19 ‡	0.63	4.26 ‡	0.69
	Peri-renal fat	4.73 ‡	0.42	4.58 ‡	0.42	4.19 ‡	0.38	4.49 ‡	0.41
	Brain	0.40 ‡	0.04	0.36 ‡	0.03	0.42 ‡	0.03	0.37 ‡	0.03
	Liver	0.71 ‡	0.13	3.19 ‡	0.12	2.72 ‡	0.10	2.64 ‡	0.12
	Right kidney	0.27	0.02	0.25	0.001	0.27	0.001	0.25	0.001
	Left kidney	0.28	0.02	0.25	0.02	0.26	0.001	0.25	0.02
	Total nitrogen	6.97	0.20	6.99	0.20	7.01	0.26	7.28	0.27
	Total fat	48.56	0.18	47.96	0.26	48.95	0.14	48.38	0.32
	Body water	45.99	0.24	46.01	0.23	46.18	0.26	45.00	0.22
Female	Gonadal fat	6.99 ‡	0.71	5.77 ‡	0.62	6.12 ‡	0.63	5.49 ‡	0.64
	Peri-renal fat	3.65 ‡	0.42	3.39 ‡	0.37	3.71 ‡	0.38	3.09 ‡	0.38
	Brain	0.59 ‡	0.03	0.59 ‡	0.03	0.62 ‡	0.03	0.58 ‡	0.03
	Liver	2.36 ‡	0.12	2.24 ‡	0.09	2.46 ‡	0.10	2.41 ‡	0.10
	Right kidney	0.28	0.001	0.26	0.001	0.24	0.001	0.26	0.001
	Left kidney	0.28	0.02	0.25	0.001	0.25	0.001	0.26	0.001

Table 5.4 Body composition of offspring exposed to cafeteria diet during lactation and post-weaning (continued)

		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex	% of body weight	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Females	Total nitrogen	6.55	0.18	6.54	0.19	6.98	0.18	6.16	0.21
	Total fat	47.98	0.27	48.13	0.31	47.65	0.29	47.76	0.35
	Body water	46.54	0.38	46.78	0.45	47.01	0.34	47.32	0.48

Table 5.4 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups exposed to cafeteria diet during lactation and weaned onto cafeteria diet. ‡ indicates the significant effect of gender ($P<0.001$).

5.4.3.2 Nutrient intakes

Cafeteria diet during the post-weaning period did not affect the daily nutritional intakes of the animals remarkably. Average energy consumption per day across the whole study was not significantly different between the groups ($P>0.05$) (Table 5.5 A and B, Figure 5.11). In addition to this, daily intakes of macro- and micro-nutrient components of the diet were not statistically different ($P>0.05$) (Table 5.5 A and B, Figures 5.12, 5.13 and 5.14). On the other hand, when the data was assessed as the consumption per kg of the body weight, exposure to pre-gestational cafeteria diet was found to significantly increase energy (male offspring: 17.6 % increase in OC and 13.6 % in OO, female offspring: % 16.1 in OC and 11.5 % in OO with respect to CC, $P<0.05$) and protein intakes (male offspring: 13.9 % increase in OC and 8.2 % in OO, female offspring: % 24.3 in OC and 15.1 % in OO with respect to CC, $P<0.05$) when compared to the pre-gestational control groups CC and CO (Table 5.5 A and B).

When the amount of chow diet consumed all through the study was analyzed between the groups, it appeared that female offspring of CO, OC and OO had significantly higher intakes of chow diet when compared to CC (interaction between pre-gestational diet, pregnancy diet and gender, $P<0.05$) (Figure 5.15). This was 52.55 % more in the CO, 73.35 % more in the OC and 38 % more in OO with respect to the CC. Male offspring did not show this effect.

5.4.3.3 Circulating lipids

When the effects of the cafeteria diet during lactation and post-weaning period combined with the maternal cafeteria diet, plasma circulating lipids did not exhibit a great disturbance. Plasma cholesterol concentrations and plasma triglyceride concentrations were similar between all groups ($P>0.05$) (Figure 5.16).

Table 5. 5(A) Average nutrient intakes of offspring exposed to cafeteria diet during lactation and and post-weaning

		Post-weaning period							
		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Males	Energy intake								
	(KJ/day/kg)	1616.32	127.71	1810.69	121.16	1901.02*	116.85	1836.28*	121.16
	(KJ/day)	387.27‡	15.16	415.94‡	15.07	381.13‡	13.78	364.37‡	14.92
	Fat intake								
	(g/day/kg)	20.84	1.78	23.11	1.69	24.11	1.63	24.06	1.69
	(g/day)	5.07‡	0.21	5.24‡	0.21	4.95‡	0.19	4.71‡	0.21
	Protein intake								
	(g/day/kg)	14.59	1.18	15.40	1.12	16.63*	1.08	15.78*	1.12
	(g/day)	3.29‡	0.14	3.47‡	0.14	3.30‡	0.13	3.12‡	0.14
	CHO intake								
	(g/day/kg)	36.53	3.62	40.69	3.49	42.71	3.30	40.08	3.44
	(g/day)	8.65‡	0.55	9.42‡	0.54	8.18‡	0.49	8.23‡	0.54
	SFA intake								
	(g/day/kg)	8.06	0.63	8.65	0.61	9.27	0.58	9.01	0.60
	(g/day)	1.96‡	0.09	2.03‡	0.09	1.96‡	0.09	1.80‡	0.09

Table 5.5 (A) Average nutrient intakes of offspring exposed to cafeteria diet during lactation and and post-weaning (continued)

		Post-weaning period							
		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Males	Sugar intake								
	(g/day/kg)	11.66‡	1.71	13.15‡	1.65	13.14‡	1.56	12.45‡	1.63
	(g/day)	2.77‡	0.25	3.06‡	0.26	2.59‡	0.23	2.46‡	0.25
	Na intake								
	(g/day/kg)	0.45	0.05	0.51	0.05	0.57	0.04	0.57	0.05
	(g/day)	0.104‡	0.005	0.113‡	0.005	0.116‡	0.005	0.093‡	0.005

Table 5.5 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. SFA: Saturated fatty acid. Na: Sodium. * indicates the significant effect of pre-gestational cafeteria diet. ‡ indicates the significant effect of gender ($P<0.001$).

Table 5.5 (B) Average nutrient intakes of offspring exposed to cafeteria diet during lactation and and post-weaning

		Post-weaning period							
		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Females	Energy intake								
	(KJ/day/kg)	1722.78	125.97	1803.98	100.61	2000.76*	116.85	1920.50*	110.59
	(KJ/day)	293.04‡	15.42	299.19‡	13.23	301.98‡	13.68	305.33‡	13.77
	Fat intake								
	(g/day/kg)	24.07	1.76	23.55	1.40	25.72	1.63	25.05	1.54
	(g/day)	4.09‡	0.21	3.82‡	0.18	3.96‡	0.19	3.95‡	0.19
	Protein intake								
	(g/day/kg)	13.87	1.16	14.69	0.93	17.24*	1.08	15.96*	1.12
	(g/day)	2.41‡	0.14	2.38‡	0.13	2.60‡	0.13	2.51‡	0.13
	CHO intake								
	(g/day/kg)	35.74	3.57	40.99	2.93	45.23	3.30	43.05	3.14
	(g/day) ‡	5.96	0.56	6.99	0.48	6.59	0.49	7.04	0.50
	SFA intake								
	(g/day/kg)	9.11	0.62	8.75	0.51	9.47	0.58	9.44	0.55
	(g/day) ‡	1.58	0.09	1.45	0.08	1.50	0.09	1.53	0.09

Table 5.5 (B) Average nutrient intakes of offspring exposed to cafeteria diet during lactation and and post-weaning (continued)

		Post-weaning period							
		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Females	Sugar intake								
	(g/day/kg) ‡	13.76	1.69	15.76	1.42	14.41	1.56	14.88	1.49
	(g/day) ‡	2.31	0.26	2.56	0.22	2.19	0.23	2.42	0.23
	Na intake								
	(g/day/kg)	0.48	0.05	0.49	0.04	0.62	0.04	0.59	0.04
	(g/day) ‡	0.078	0.006	0.080	0.005	0.079	0.005	0.083	0.005

Table 5.5 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. SFA: Saturated fatty acid. Na: Sodium. * indicates the significant effect of pre-gestational cafeteria diet. ‡ indicates the significant effect of gender ($P<0.001$).

Figure 5. 11 Average energy intakes of male and female offspring exposed to cafeteria diet during lactation and post-weaning

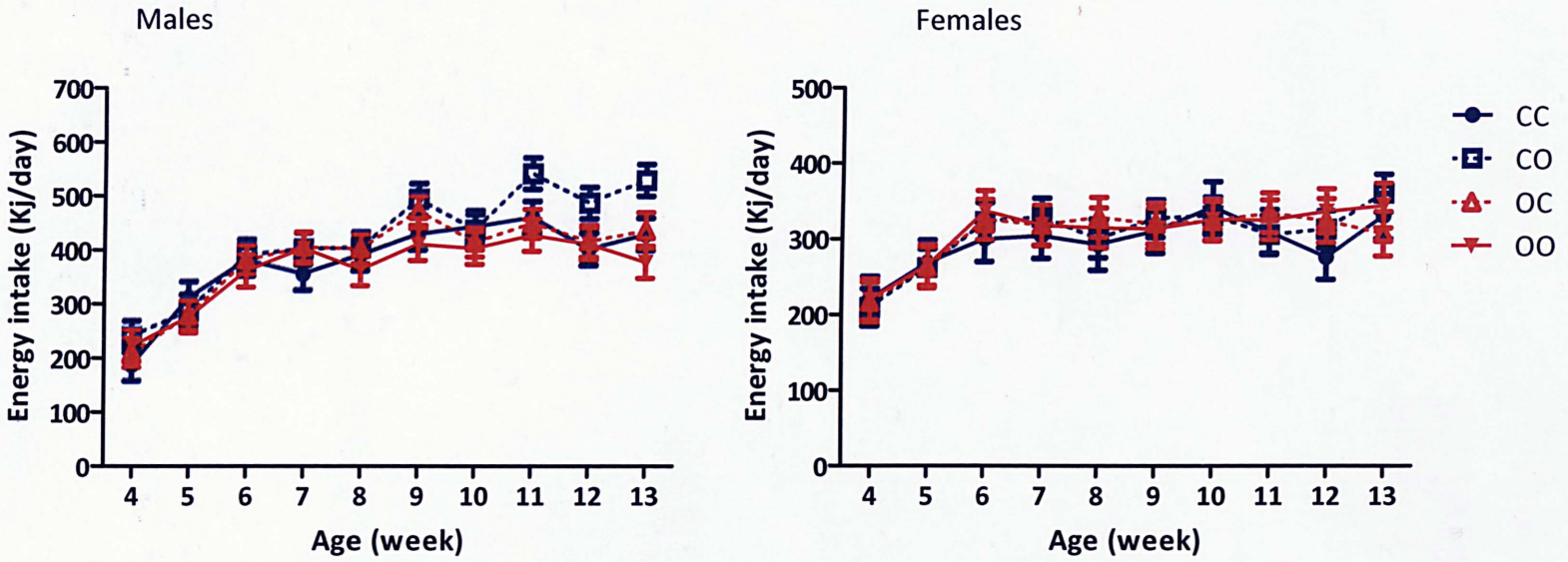


Figure 5.11 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. The effect of gender and study weeks were significant (P<0.001).

Figure 5. 12 Average protein intakes of male and female offspring exposed to cafeteria diet during lactation and post-weaning

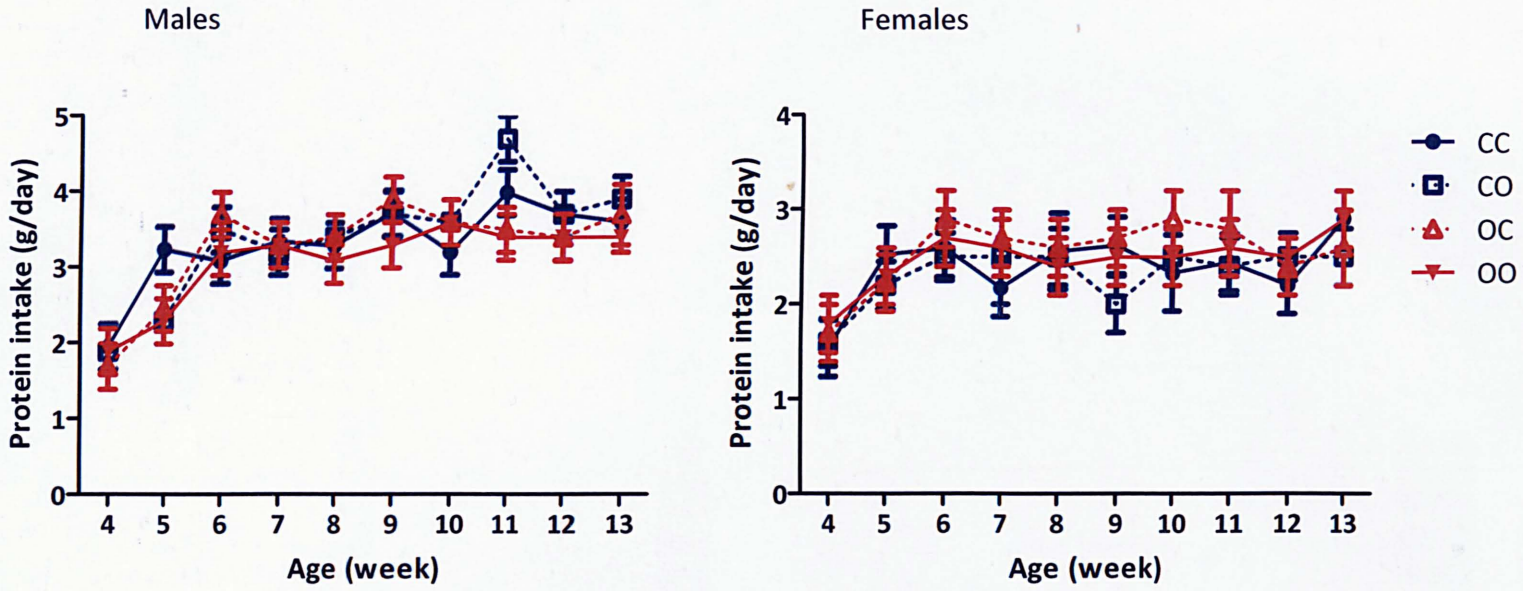


Figure 5.12 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. The effect of gender and study weeks were significant (P<0.001).

Figure 5. 13 Average fat intakes of male and female offspring exposed to cafeteria diet during lactation and post-weaning

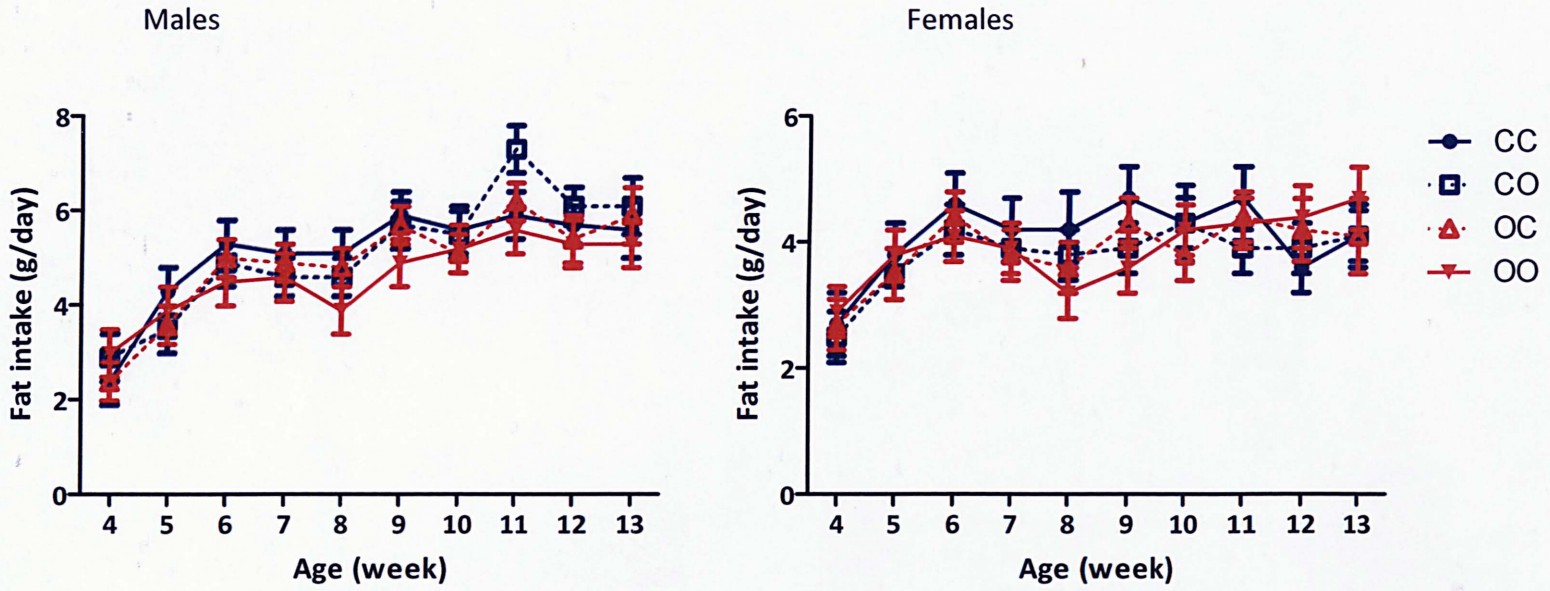


Figure 5.13 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. The effect of gender and study weeks were significant (P<0.001).

Figure 5. 14 Average carbohydrate intakes of male and female offspring exposed to cafeteria diet during lactation and post-weaning

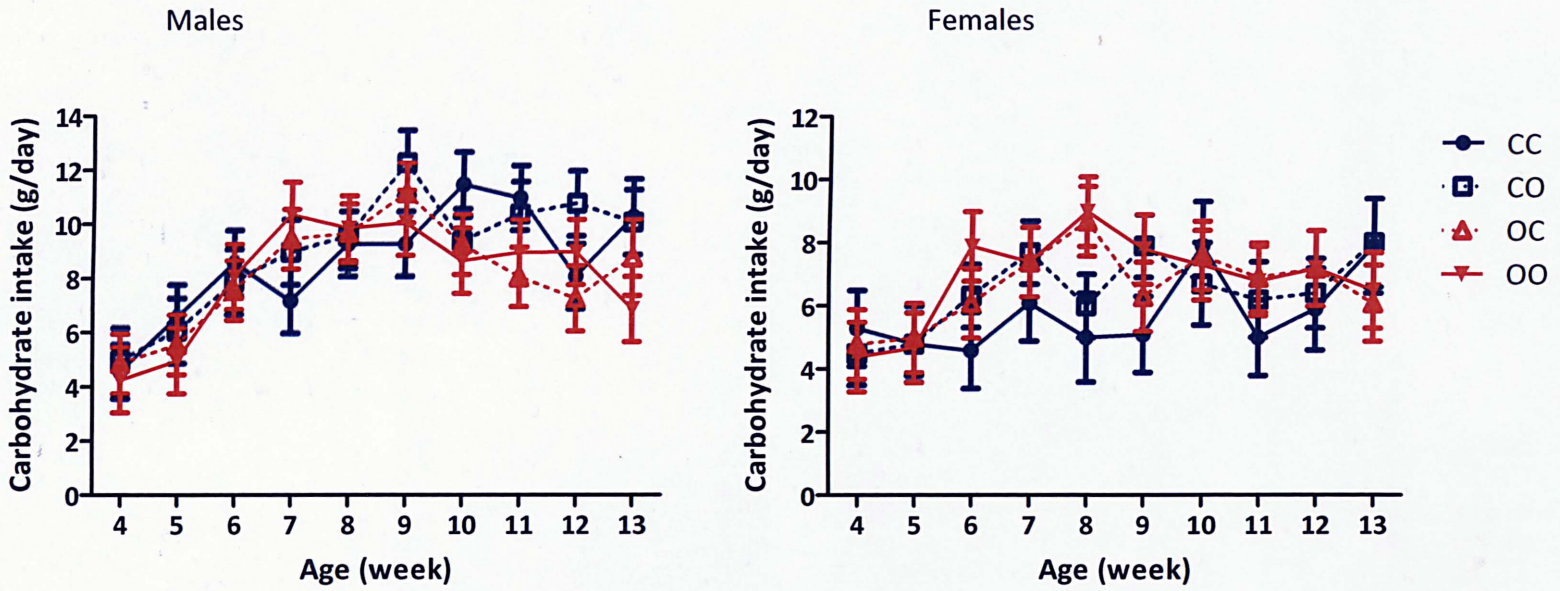


Figure 5.14 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. The effect of gender and study weeks were significant (P<0.001).

Figure 5. 15 Average percentage of chow diet energy in male and female offspring exposed to cafeteria diet during lactation and post-weaning

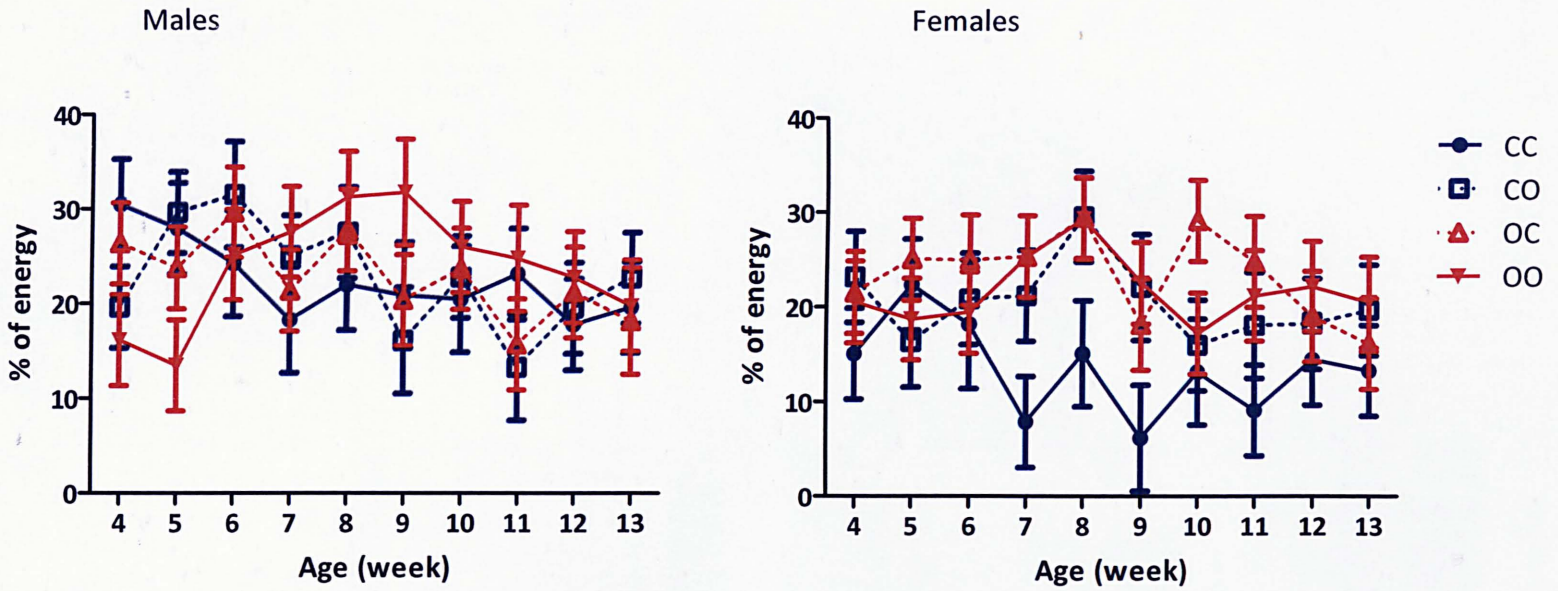


Figure 5.15 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. Female offspring of OC and CO had significantly higher intakes of chow diet ($P<0.05$).

Figure 5. 16 Plasma cholesterol and triglycerides of offspring exposed to cafeteria diet during lactation and post-weaning

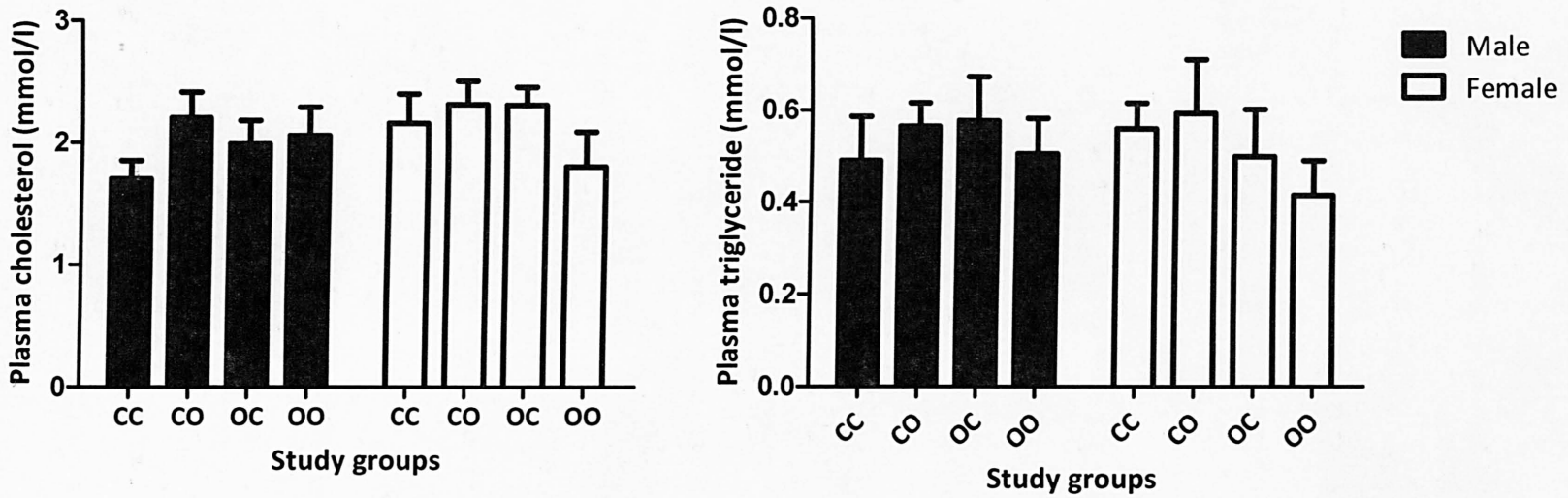


Figure 5.16 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet.

5.4.3.4 Glucose homeostasis

Permanent cafeteria feeding from the beginning of postnatal life resulted in marked disturbance of glucose homeostasis. Baseline fasting glucose and insulin concentrations were similar between all groups (Table 5.6). Insulin concentrations half an hour after the glucose injection were significantly lower in the groups exposed to pregnancy cafeteria diet (CO and OO) than in the pregnancy chow diet groups (CC and OC) ($P<0.05$) (Table 5.6). Nevertheless, Δ Insulin concentrations were similar between all groups (Table 5.6).

When the glucose response to intraperitoneal load was assessed, from the thirtieth minute to the end of the tolerance test, male offspring of CO and OC had higher plasma glucose concentrations than CC controls whereas female offspring of these groups had lower concentrations than controls (significant interaction between pre-gestational diet, pregnancy diet and gender, $P<0.05$) (Figure 5.17). Male offspring of CO and OC groups had remarkably higher areas under the glucose curve (significant interaction between pre-gestational diet, pregnancy diet and gender, $P<0.05$) (Table 5.6).

Expression of components of the insulin signalling pathway data partially supported these observations. IRS2 mRNA expression in liver was similar between all groups ($P>0.05$) (Figure 5.18). However, AKT2 mRNA expression tended to be lower in the males of CO and OC groups (non-significant interaction between gender, pre-gestation and pregnancy diet, $P=0.07$) (Figure 5.18).

Table 5. 6 Glucose tolerance data of offspring exposed to cafeteria diet during lactation and post-weaning

		CC (n=4)		CO (n=4 or 6)		OC (n=5)		OO (n=4 or 5)	
Sex		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male	Baseline glucose (mg/ml)	0.68‡	0.08	0.72‡	0.07	0.82‡	0.07	0.76‡	0.08
	Baseline insulin (ng/ml)	1.59	0.36	2.14	0.36	1.64	0.32	1.28	0.36
	Insulin at 30 min (ng/ml)	2.97‡	0.44	2.59‡†	0.44	3.46‡	0.39	1.90‡†	0.44
	Δ insulin (ng/ml)	1.22	0.47	1.06	0.67	1.92	0.42	1.25	0.55
	AUC glucose (mg/ml.min)	134.82	27.52	184.20 ¥	27.52	185.30‡¥	24.61	135.38	27.52
Female	Baseline glucose (mg/ml)	0.69‡	0.07	0.75‡	0.08	0.66‡	0.07	0.69‡	0.07
	Baseline insulin (ng/ml)	0.69	0.36	1.14	0.36	0.71	0.32	0.35	0.40
	Insulin at 30 min (ng/ml)	2.34‡	0.39	1.15‡†	0.51	2.46‡	0.39	2.18‡†	0.39
	Δ insulin (ng/ml)	1.91	0.47	0.76	0.67	1.87	0.42	1.73	0.42
	AUC glucose (mg/ml.min)	149.63	24.61	139.26	27.52	112.96	24.61	163.23	24.61

Table 5.6 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation, CO: Chow diet during pre-gestation and cafeteria diet during gestation, OC: Cafeteria diet during pre-gestation and chow diet during gestation, OO: Cafeteria diet during pre-gestation and gestation. All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. Δ Insulin: the difference between baseline and thirty minute insulin concentrations. † indicates the significant of gestational cafeteria diet ($P<0.05$). ¥ indicates the significant interaction between pre-gestational diet, gestational diet and gender ($P<0.05$). ‡ indicates the significant effect of gender ($P<0.05$).

Figure 5. 17 Plasma glucose concentrations following intraperitoneal glucose tolerance test of offspring exposed to cafeteria diet during lactation and post-weaning

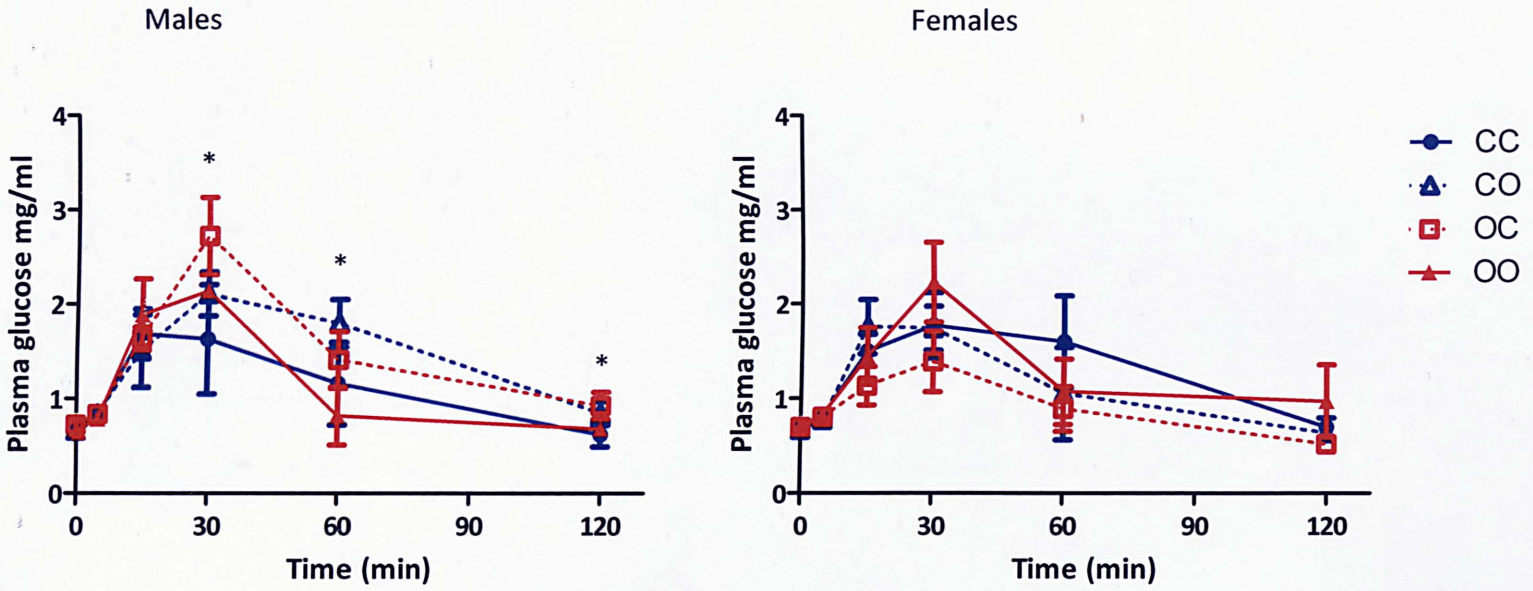


Figure 5.17 Data is shown as mean±SEM. Data was analysed at each time point separately for the effects of gender, pre-gestational and pregnancy diets. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet. * indicates the significant interaction between gender, pre-gestational and pregnancy diets.

Figure 5. 18 Expression of genes in the insulin signalling pathway of offspring exposed to cafeteria diet during lactation and post-weaning

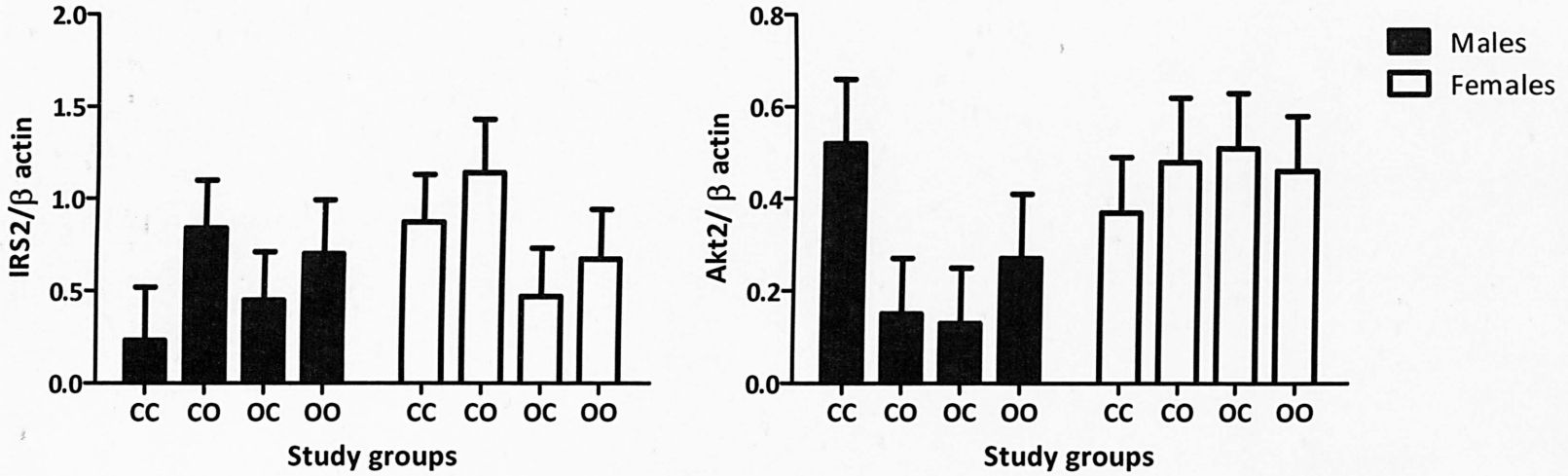


Figure 5.18 Data is shown as mean±SEM. CC: Chow diet during pre-gestation and gestation (n=4 for males and females), CO: Chow diet during pre-gestation and cafeteria diet during gestation (n=4 for males and n=6 for females), OC: Cafeteria diet during pre-gestation and chow diet during gestation (n=5 for males and females), OO: Cafeteria diet during pre-gestation and gestation (n=4 for males and n=5 for females). All groups were exposed to cafeteria diet during lactation and weaned onto cafeteria diet.

5.4.4 Comparison of the Effects of Chow Diet versus Cafeteria Diet During Lactation on Offspring When the Post-Weaning Diet is Cafeteria

As discussed in Section 4.4.4 an interaction between pregnancy and lactation diets showed that the offspring of XCO (X: C or O pre-gestational diet) and XOC (X: C or O pre-gestational diet) had significantly lower body weights (Tables 4.1 and 4.8). When the average body weights during the post-weaning period were analyzed it was apparent that the female offspring of the animals fed cafeteria diet during lactation had significantly lower body weights than chow diet fed animals during lactation (194.49 ± 1.72 g in C during lactation versus 184.93 ± 1.84 g in O during lactation, interaction between diet during lactation and gender, $P<0.05$). This effect was not observed in male offspring. There was also a further interaction between gender, pre-gestational, pregnancy and lactation diets implying that the body weights were influenced by cafeteria diet at any stage of the study when the post-weaning environment was cafeteria diet. Dietary treatment during lactation had a significant effect on peri-renal fat pad mass as the offspring fed by cafeteria diet during lactation had significantly greater peri-renal fat pad mass than offspring fed by chow diet during lactation (male offspring: 3.67 ± 0.18 % of body weight in C during lactation versus 4.34 ± 0.19 % of body weight in O during lactation; female offspring: 3.26 ± 0.17 % of body weight in C during lactation versus 3.38 ± 0.17 % of body weight in O during

lactation, P<0.05). However, gonadal fat pad mass and total body fat content was not influenced by the dietary treatment during lactation.

Male offspring fed cafeteria diet during lactation had significantly higher energy intake than chow diet fed male offspring (355.21 ± 4.70 kj/day in C during lactation versus 382.67 ± 4.81 kj/day in O during lactation, P<0.05). This effect was not observed in female offspring. Energy intakes per kg of body weight were not influenced by the dietary treatment during lactation.

Plasma cholesterol concentrations tended to be higher in offspring of rats fed cafeteria diet during lactation than chow diet fed offspring during lactation (Male offspring: 1.71 ± 0.11 mmol/l in C during lactation versus 1.99 ± 0.14 mmol/l in O during lactation; female offspring: 1.98 ± 0.16 mmol/l in C during lactation versus 2.15 ± 0.12 mmol/l in O during lactation, P=0.05). Plasma triglyceride concentrations were not influenced by the dietary treatment during lactation.

When the post-weaning environment was cafeteria diet, fasting insulin and glucose concentrations were similar between the groups fed chow and cafeteria diet during lactation. On the other hand, Δ insulin (the difference between baseline and 30 minutes post-injection plasma insulin concentration) was significantly lower in animals fed cafeteria diet during lactation than in chow diet animals (male offspring: 1.74 ±0.36 ng/ml in C during lactation versus 1.36 ± 0.36 ng/ml in O during lactation; female offspring 2.84 ± 0.30 ng/ml in C during lactation versus 1.57 ± 0.34 ng/ml in O

during lactation). This may suggest that the offspring whose mothers were fed by cafeteria diet during lactation had relative insulin deficiency when compared to control animals. However, dietary treatment during lactation did not influence the area under glucose curve data. IRS2 mRNA expression data showed an interaction between gender, dietary treatments of pregnancy and lactation. With respect to the male offspring of the CCC (0.21 ± 0.27 βactin/IRS2), OCC (0.99 ± 0.22 βactin/IRS2), COC (0.62 ± 0.24 βactin/IRS2), OOC (0.37 ± 0.26 βactin/IRS2), CCO (0.23 ± 0.29 βactin/IRS2) and OCO (0.44 ± 0.24 βactin/IRS2) had significantly upregulated mRNA expression of IRS2 ($P<0.05$). AKT2 mRNA expression data exhibited a four-way interaction between gender, pre-gestational, pregnancy and lactation diets which suggested that it was influenced by cafeteria diet at any stage of the study ($P<0.05$).

5.4.5 Summary of the findings

Table 5. 7 Summary of the findings when the offspring were exposed to chow diet during lactation and cafeteria diet during post-weaning

	Effects of Cafeteria Diet	
	Pre-gestation	**Pregnancy**
Birth weight	↓	↑
Weaning weight	↔	↓
Post-weaning body weight	↓ (males)	Interaction ↓ (males)
Gonadal fat pad	↔	↔
Peri-renal fat pad	↔	↔
Total body fat	↔	↔
Brain	↔	↔
Liver	↔	↔
Left kidney	↔	↔
Right kidney	↔	↔
Energy intake (kj/day)	↓	↔
Energy intake (kj/day/kg)	Interaction with gestation ↑	↑
Plasma cholesterol	↔	↔
Plasma triglyceride	↔	↓
Baseline glucose	↔	↔
Baseline insulin	↔	↓ (males)
30 min post glucose injection insulin	Interaction ↑	
Δ insulin	↑ (females)	↔
AUC for glucose	Interaction ↑	
mRNA expression of IRS2	Interaction ↑	
mRNA expression of AKT2	↔	↔

Table 5.7 shows a brief summary of results from animals exposed to chow diet during lactation and cafeteria diet during post-weaning. Early life cafeteria diet resulted in glucose intolerance with insulin resistance.

Table 5. 8 Summary of the findings when the offspring were exposed to cafeteria diet during lactation and post-weaning

	Effects of Cafeteria Diet	
	Pre-gestation	**Pregnancy**
Birth weight	↓	↑
Weaning weight	↔	↑
Post-weaning body weight	↔	↔
Gonadal fat pad	↔	↔
Peri-renal fat pad	↔	↔
Total body fat	↔	↔
Brain	↔	↔
Liver	↔	↔
Left kidney	↔	↔
Right kidney	↔	↔
Energy intake (kj/day)	↔	↔
Energy intake (kj/day/kg)	↑	↔
Plasma cholesterol	↔	↔
Plasma triglyceride	↔	↔
Baseline glucose	↔	↔
Baseline insulin	↔	↔
30 min post glucose injection insulin	↔	↓
Δ insulin	↔	↔
AUC for glucose	Interaction ↑ (males)	
mRNA expression of IRS2	↔	↔
mRNA expression of AKT2	↔	↔

Table 5.8 shows a brief summary of results from animals exposed to cafeteria diet during lactation and post-weaning. Early life cafeteria diet resulted in glucose intolerance with decreased insulin response.

5.5 Discussion

In the current study, the effects of maternal obesity and/or maternal cafeteria diet on offspring body composition and glucose handling health status were studied when the post-natal feeding regime was cafeteria diet. In the previous chapter, when the post-natal dietary treatment was chow diet, offspring of cafeteria diet fed rats exhibited an improved glucose tolerance which was characterised by lower fat pads and normal feeding behaviour. One of the main aims of this part of the trial was to examine the programmed offspring in an obesogenic environment in order to observe whether this additional metabolic load during post-natal life would result in a different phenotype. Indeed, 10 weeks of cafeteria diet during post-weaning period after early life cafeteria diet resulted in a different phenotype than what was observed in Chapter 4. Our data in the current chapter suggested that, when cafeteria diet exposure in early life was combined with post-natal cafeteria diet, offspring became glucose intolerant (Tables 5.7 and 5.8). More specifically, the mechanisms involved in this outcome were shown to be dependent on the dietary treatment during lactation (Tables 5.7 and 5.8). It is particularly noteworthy that the offspring of the group OO that had cafeteria diet during suckling and were exposed to cafeteria diet throughout their life exhibited normal growth rates in both genders when compared to control group. This suggests that the cafeteria diet must therefore deliver the nutritional requirements.

The nutritional data from this arm of the offspring trial were similar to those reported in Chapter 4. When the offspring of obese and/or cafeteria diet fed rats were weaned on to cafeteria diet during post-natal life, body composition, body weight, food intake and preferences were not altered in the manner that had been expected on the basis of published studies (Tables 5.7 and 5.8). Previously Bayol *et al.,* reported that the offspring of rats fed cafeteria diet during gestation and suckling developed an exaggerated preference for the cafeteria diet foods, rather than chow diet, when compared to control animals *(Bayol et al., 2007).* The findings of the present study were very different to this report. Firstly, in parallel with data from the previous chapter, offspring of obese mothers (pre-gestational cafeteria diet groups) exhibited lower body weights throughout the post-natal study, which may suggest growth retardation, but this effect disappeared with cafeteria diet during lactation (Tables 5.7 and 5.8). Secondly, feeding cafeteria diet only during pregnancy did not result in increased hyperphagia and adiposity in the offspring (Tables 5.7 and 5.8). Energy intakes per kg of body weight were increased in both pre-gestational and pregnancy cafeteria diet exposed groups but this did not trigger any increase in adiposity in these animals (Tables 5.7 and 5.8). In fact, when the food preferences of the groups were analyzed a surprising interaction between pre-gestational and gestational diets was observed, indicating that the female offspring of the groups CO and OC consumed more chow diet than the other groups. Programming of taste preference was shown by Bellinger and Langley-Evans in a different study where female offspring of low protein diet fed rats consumed less fat when

the nutrient preference was given *(Bellinger and Langley-Evans, 2005).* It is very difficult to explain the underlying cause of this behaviour, but it may suggest an attempt for the offspring in the current study to obtain the necessary nutrients from a low fat chow diet when a choice was given. More specifically, when the offspring of cafeteria diet fed animals only during lactation were assessed by behavioural satiety sequence (BSS), female offspring were shown to have significantly later satiety (long period of eating), more resting time and increased serotonin levels in hypothalamus when compared to offspring of chow diet fed animals *(Wright and Langley-Evans, manuscript under preparation).* For this reason, these data suggest that exposure to cafeteria diet during neo-natal or early life may result in altered feeding behaviour at the hypothalamic level. Therefore, assessing the hypothalamus and neurotransmitters that are involved in feeding control of these offspring is an important point for future study.

Despite using very similar study designs, our data showed different outcomes to what Bayol *et al.,* reported previously. As discussed in Chapter 4, offering rats a pre-gestational dietary regime for 8 weeks may be one of the main reasons for the difference in results in the current study, since Bayol *et al.,* did not include this pre-gestational regime in their study *(Bayol et al., 2007).* In addition to this, in our offspring trials litter size was reduced to 8 pups (4 male and 4 female) shortly after birth to minimize the milk intake difference between litters, whereas Bayol et al., kept each litter in their original size *(Bayol et al., 2007).* It was shown by Schmidt et al., that

overfeeding during the suckling period in rodents, by rearing them in smaller litters may produce hyperphagia and obesity *(Schmidt et al., 2001).* Therefore, keeping litter sizes in different numbers in programming studies may result in different offspring phenotype. When compared to other cafeteria diet trials, the energy intake of the dams in our trial was found to be relatively lower *(Akyol et al., 2009, Shafat et al., 2009, Bayol et al., 2007).* Since maternal hyperphagia was considered as an important factor for programming of obesity in offspring, the rates in the current study may not be enough to induce hyperphagia in resulting offspring. However, in our maternal trial, it was shown that pre-gestational or pregnancy cafeteria diet resulted in marked adiposity *(Akyol et al., 2009).*

It was clear from this arm of the offspring trial that disturbances of glucose homeostasis, which were largely absent when rats were weaned onto chow, manifested in several groups when weaned onto the hypercaloric cafeteria diet. Analaysis of the area under the glucose curve data suggested that, compared to CCC controls, several groups of offspring were glucose intolerant. Interestingly, our data suggested that this could happen through different mechanisms depending on the timing of exposure to cafeteria diet. Offspring of groups COC and OCC exhibited insulin resistance as their plasma glucose concentrations remained higher during the intraperitoneal glucose tolerance test despite increased plasma insulin concentrations. In males of group COC the insulin response was exaggerated as the fasting concentration was low. The insulin resistance of COC males, characterized by a strong insulin

response to glucose tolerance test, could be the product of a defect of insulin signalling. In our study expression of two genes involved in the insulin signalling pathway in liver, IRS2 and AKT2, was measured. Although there was limited evidence of markedly perturbed insulin signalling pathways which favoured insulin resistance, the up regulation of IRS2 in groups COC and OCC may be an indicator of an alteration in the insulin signalling mechanisms. Other components of insulin signalling pathway such as insulin receptor and glucose transporter (GLUT 4) in downstream of phosphatidylinositol 3-kinase may also be altered and may play an important role for the observed outcomes. Up-regulation of insulin signalling mechanisms were also shown in another study with general induction of insulin responsive lipogenic pathways *(Shankar et al., 2010).* Bayol *et al* reported the changes in the regulation of glucose transporters and insulin receptor in liver with hepatic steatosis and increased oxidative stress characterized by increased hepatic glucose uptake in the offspring of cafeteria diet fed dams *(Bayol et al., 2010).*

Insulin signalling may not have been the only mediator of impaired glucose tolerance in the programmed offspring. Rats of group COO and male offspring of OCO showed higher plasma glucose concentrations after glucose tolerance test, with evidence of an impaired insulin response in group COO. This may suggest a pancreatic defect or insufficiency for these animals. It is noteworthy that in our study, these observations of impaired glucose homeostasis were not related to increased adiposity or hyperphagia. To our

knowledge, this is the first study showing effects of maternal cafeteria diet feeding on glucose homeostasis which are independent of these factors.

Pancreatic β-cell numbers are regulated by the balance between β-cell replication and apoptosis *(Bonner-Weir, 2000).* Development of new islets from pancreatic ducts by neogenesis also play important role *(Finegood et al., 1995).* Disruption of any of the pathways of β cell formation, or increased rates of β cell apoptosis would result in decreased β cell mass, therefore reduced capacity of insulin production *(Leonardi et al., 2003).* Cerf *et al.,* demonstrated that high fat feeding during pregnancy caused compromised β cell development in neonates and increased duration of high fat feeding during pregnancy elicited a greater effect *(Cerf et al., 2005).* Samuelsson *et al.,* reported that the insulin resistance observed in the offspring of overfed mice was associated with pancreatic β cell exhaustion *(Samuelsson et al., 2008).* However insulin resistance with normal pancreatic insulin secretion was also reported in a similar study design *(Nivoit et al., 2009).*

The final week of gestation is the most quantitavely important phase of islet histogenesis (*Bouwens and Kloppel, 1996).* It is reported that the rat pancreas starts to develop during the second week and the endocrine pancreas in the final week of pregnancy *(Cerf et al., 2005).* In low protein diet fed neonates β-cell proliferation, islet size and vascularisation was shown to decrease by the end of pregnancy *(Snoeck et al., 1990).* If those offspring were kept on low protein diet during suckling and post-weaning fasting insulin concentrations remained lower with normal blood glucose concentrations

(Dahri et al., 1991). On day 70 of post-weaning period, glucose tolerance impaired with decreased insulin response *(Dahri et al., 1991).* Our data suggested that pancreatic capacity of insulin secretion could be influenced by the dietary treatment during lactation since we observed the signs of glucose intolerance due to insufficient insulin secretion mostly in the animals exposed to cafeteria diet during lactation. Hyperglycaemia is a possible causative factor for the deterioration of β-cells and resulting loss of insulin secretory capacity in rats fed a high energy diet *(Jorns et al., 2002).* Exposure of islets to high circulating glucose concentrations for an extended period resulted in relative loss of their regenerative potential in hyperglycaemia induced mice *(Guz et al., 2002).* Furthermore, exposure to a maternal high fat diet during lactation only was shown to be sufficient to induce hyperglycaemia in weanling offspring *(Cerf et al., 2006).* It is possible therefore, that the hyperglycaemia observed in the groups COO and OCO may have inhibited islet neogenesis. These data suggest that the first few weeks of post-natal life could be a vulnerable period for the pancreas. Previously, the milk composition of cafeteria diet fed dams were shown to be altered with increased energy, fat, sugar and decreased protein *(Rolls et al., 1986).* In addition to this, during the final week of lactation consumption of cafeteria diet foods by pups was observed during the trials. For these reasons, the increased intake of glucose and energy by the pups may explain the vulnerability of the pancreas during lactation. Despite these important findings, there are a few limitations in the current trial that need to be addressed in future studies. Measurement of plasma glucose and insulin

concentrations earlier in life, for instance shortly after birth and during lactation, and histological assessment of pancreas from these offspring could introduce a broader explanation of the mechanistic basis of these outcomes.

In conclusion, our data showed that glucose intolerance could be programmed in offspring of cafeteria diet fed rats if challenged with cafeteria diet from weaning. This occurred through different mechanisms, depending on the timing of maternal over-feeding insults. One of the main aims of this study was to address the individual effects of maternal obesity or cafeteria diet feeding during pregnancy only. Despite the interactions between these two influences, our data suggested that depending on the dietary treatments during lactation, maternal obesity (pre-gestational cafeteria diet) may tend to programme glucose intolerance and insulin resistance through altered insulin signalling pathways, whereas cafeteria diet during lactation may impact upon pancreatic development. When taken together, it is clear that the additional metabolic load of cafeteria diet during post-weaning period bring out the hidden phenotype of chow diet during post-weaning of the cafeteria diet exposed offspring during neo-natal life.

6.0 GENERAL DISCUSSION and FUTURE PROSPECTS

Nutritional status during critical periods of early life is a crucial factor in the normal growth and development of the organism and maturation of metabolic systems. In this context, the fetal programming hypothesis states that sub-optimal nutritional conditions *in utero* lead to permanent alterations in tissue structure, function and metabolism. These adaptations predispose to complex chronic disease later in life. Early epidemiological observations built up this concept by showing that geographical variation in coronary heart diseases was correlated with past variation in neonatal death rates. These associations were mostly attributable to low birth weight *(Rich-Edwards et al., 1997, Leon et al., 1998).*

Hales and Barker extended this link, developing the thrifty phenotype hypothesis which suggests that poor nutrition in fetal life can lead to permanent changes in the glucose-insulin axis *(Hales and Barker, 2001).* However, the generalisability and relevance of this hypothesis has been questioned, since a meta-analysis showed that the effect was minimal at the population level *(Huxley et al., 2002).* Animal study models contributed to a further understanding of these phenomena. Although earlier studies were based on undernutrition in pregnancy, given contemporary trends it is necessary to investigate the long-term outcomes of maternal over nutrition and obesity. With evidence of increasing consumption of energy dense foods and a significant rise in over-weight and obesity in child-bearing age women, this subject is of great importance *(Gallou-Kabani and Junien, 2005).*

A body of evidence suggests that a fat rich diet during rodent pregnancy may induce the characteristics of metabolic syndrome in the adult offspring *(Guo and Jen, 1995, Napoli and Palinski, 2001).* Most of the commercial laboratory chow diets for rodents contain 4 % to 6 % dietary fat. Chow formulations are intended to prevent the development of obesity in laboratory rodents and are based upon a level of 5 % dietary fat that was set as an adequate amount for growth and maintenance of rats *(National Research Council, 1995).* High fat diet studies that have assessed the relationship between diet induced obesity during pregnancy and resulting health status of offspring, have used several different fat contents and types varying between 34 and 60 % fat. Saturated fat was often incorporated as the primary source of fat *(Ainge et al., 2010).*

In the current study, the decision was made to utilise cafeteria feeding, as originally developed by Rothwell and Stock (1979), to induce and maintain maternal obesity. The basis for this decision was that, despite variation in actual foods consumed between animals, the obesity associated with cafeteria feeding is driven by the same principles as human obesity, i.e. excess availability of a diverse range of highly palatable and energy dense foods. Cafeteria diet resulted in consumption of a diet that was 53 % of fat with associated decreases in protein and carbohydrate proportions. This caused a profound obesity in mothers. Although offspring trials in this study did not demonstrate a marked effect of maternal cafeteria diet in terms of adiposity and hyperphagia, offspring which were weaned onto cafeteria diet

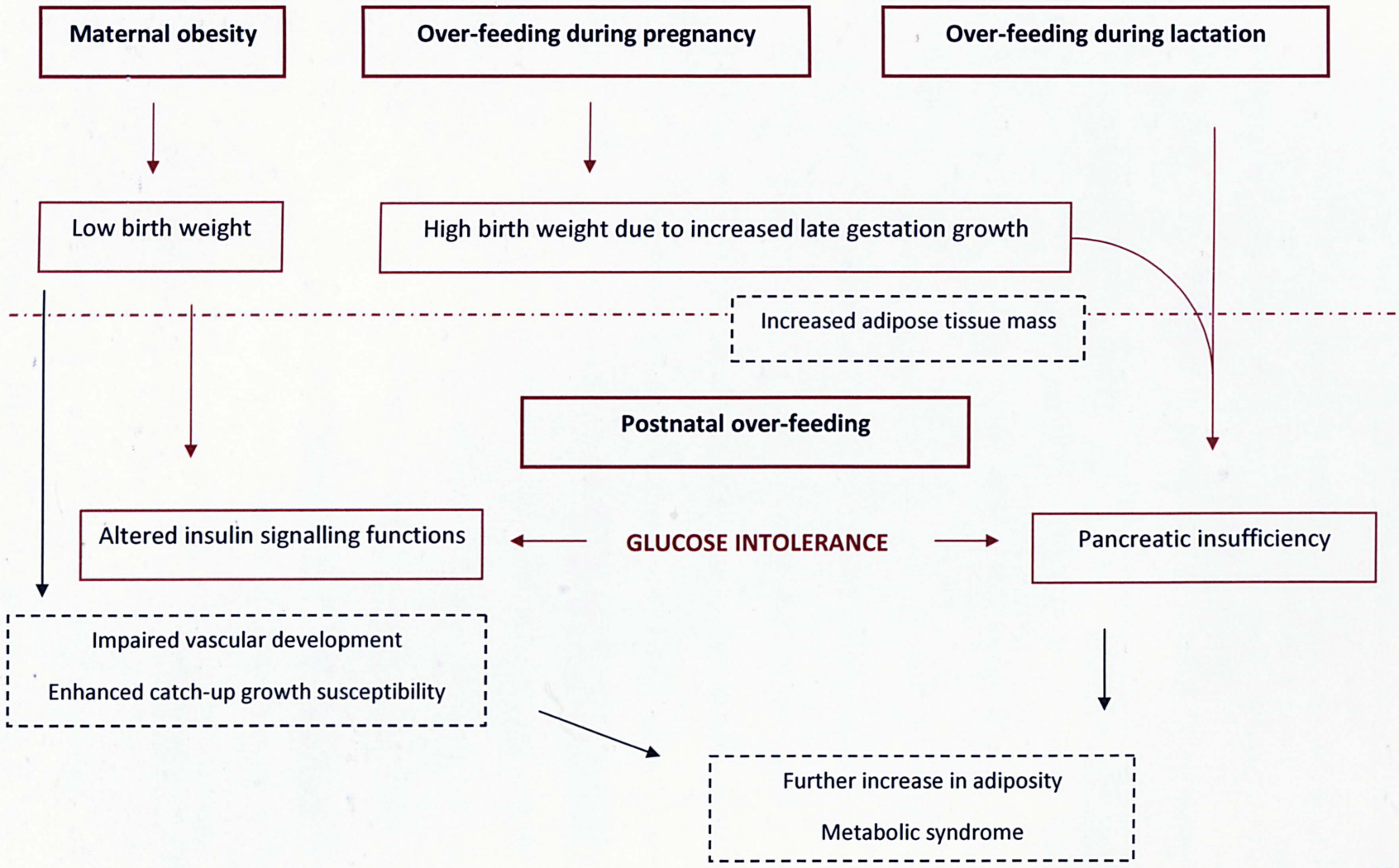
weighed significantly more, had increased plasma triglyceride concentrations and larger fat depots than chow fed controls. Despite representing a model for high-fat diet, cafeteria diets also provide considerably higher salt and sucrose intake and a lower protein intake. Hence, the observed effects of maternal cafeteria diet feeding could be attributable to the combined features of any or all of these characteristics of the diet. The feeding of a cafeteria diet to rats demonstrated a useful method for characterizing the impact of a non-prudent human diet in animal studies, without impacting on reproductive abilities, litter size and physical adaptation to pregnancy.

One of the main aims of this study was to identify the independent effects of maternal adiposity and maternal diet on the growth, development and glucose homeostasis of offspring. We introduced a pre-gestational dietary treatment which gave the opportunity to switch diets between the groups. Hence the group OC represented maternal obesity at conception independently of diet during pregnancy and CO represented the effects of the diet from conception only independently of the effect of obesity. In order to clarify the influence of each period, additional treatments were added into study for the suckling and postnatal environments. This resulted in a very complex study design; however this comprehensive study design was thought to be essential to elucidate the possible mechanisms associated with offspring health status. Certain metabolic processes can be programmed at critical periods of fetal and early life through conferring a survival phenotype *(Cottrell and Ozanne, 2007).* These adaptations have been thought to be beneficial if

the suboptimal environment experienced during fetal life continues to operate postnatally *(Hales and Barker, 1992).* However, if the postnatal environment provides plentiful nutrition, these offspring would be at increased risk of developing diseases such as obesity and type 2 diabetes *(Hales and Barker, 1992).* Although our data exhibited some interactions between the maternal diets, clues to the effects of specific periods were noted. Figure 6.1 represents a summary of the findings in the context of the current literature.

Studies of rodent models of maternal high fat feeding report variable effects upon birthweight. Some studies reported no effect (Khan et al., 2003, Gorski et al., 2006), while others reported either decreased (Hausman et al., 1991, Taylor et al., 2003) or increased birth weights (Samuelsson et al., 2008). In our study, differential effects of maternal obesity and diet were exhibited. Maternal obesity at pre-conception (with or without cafeteria feeding during pregnancy) decreased birth weights, an effect which was independent of placental growth. In contrast cafeteria diet during pregnancy only led to heavier birth weights, which appeared to be induced through rapid growth during the last two days of pregnancy. This data may suggest a macrosomic outcome of hyperglycaemic pregnancies. Indeed, the maternal group CO exhibited greater plasma glucose concentrations at day 20 of gestation.

Figure 6. 1 Summary of the findings in context of literature



Previously it was shown that macrosomic offspring born to diabetic mothers were prone to the development of glucose intolerance and obesity as a function of age in rats *(Khan, 2007).* This relationship was also established in several human studies. Babies in pregnancies complicated by gestational diabetes and high body mass index were larger at birth, showed increased adipose tissue mass and obesity and diabetes risk in later life *(Dang et al., 2000, Ehrenberg et al., 2004, Gillman et al., 2003, McCance et al., 1994, Parsons et al., 2001).*

In terms of programming of obesity, the data in this study exhibited a number of outcomes which were not consistent with the most frequently cited literature in this field *(Bayol et al., 2007, Bayol et al., 2008, Samuelsson et al., 2008, Shankar et al., 2008).* Rather than inducing adiposity and hyperphagia, pre-gestational cafeteria diet was associated with growth retardation and glucose intolerance. The driving mechanism underlying this observation is unknown. Other animal studies in this area presented data that shared some similarities with the current work. For instance, no evidence of greater food consumption in the offspring of animals fed high fat diets was reported by Howie et al., (2009) and Dyrskog et al., (2005). Gregersen *et al.,* reported that high fat feeding before and during pregnancy did not induce increased body weight in the resulting male offspring (*Gregersen et al., 2005*). These studies also observed no effects of maternal diet on glycaemic control when the offspring were 3 months old, in contrast to the current study *(Howie et al., 2009, Dyrskog et al., 2005).* Maternal obesity was shown to be

the sole driver of adiposity but no evidence of metabolic dysfunction was associated with maternal condition *(White et al., 2009).*

In the absence of consistent observations, it is difficult to begin to identify the mechanistic factors which may be responsible for mediating the effect of maternal obesity on metabolic function in the offspring. The variability of fat composition between the high-fat diets used in different studies may be the factor responsible for these inconsistent results. It was reported that, traditionally, developing high-fat test diets consists of a simple exchange between carbohydrate and fat compositions when compared to chow diet *(Kennedy et al., 2010).* The increase in fat content is supplied by lard in most of the studies of early programming, a choice driven by the higher content of saturated fatty acids *(Armitage et al., 2005, Khan et al., 2005, Taylor et al., 2003, Farnworth and Kramer, 1990).* However, despite higher content of saturated fatty acids, lard is also rich in mono-unsaturated fatty acids *(Yepuri et al., 2011).* Bouanane et al., evaluated the fatty acid composition of cafeteria diet and reported that 42 % of total fat was saturated, 33 % was monosaturated and 25 % was polyunsaturated fatty acids *(Bouanane et al., 2010).* The ratio of omega 6 to omega 3 was found to be 2.33 whilst this ratio was 0.56 in chow diet *(Brunetti et al., 2010).* For this reason, using cafeteria diet models is considered to reflect the variety of human diets more accurately since it delivers a range of fatty acids that are prevalent in Western society, rather than specific classes of fatty acids alone

and in excessive quantities *(Rothwell and Stock, 1979, Sclafoni et al., 2008, Sampey et al., 2011).*

Interestingly, the current work demonstrated glucose intolerance, independent of increased food intake and body adiposity. Both pancreatic insufficiency (female offspring of COO and OOC) and impaired insulin signalling (male offspring of COC and OCO) were noted but the metabolic impact of this was dependent on the post-weaning nutritional environment. Indeed, when the postnatal nutritional environment was low fat chow diet, offspring of maternal cafeteria diet groups exhibited an improved insulin response, whereas in an obesogenic environment this scenario reversed. Studies in adult rats have shown that high-fat feeding resulted in increased glucose concentrations, reduced insulin concentrations and induced insulin resistance *(Kim et al., 1995, Winzell and Ahren, 1999).* It is clear from the current data that the programmed offspring from cafeteria diet fed mothers could not tolerate the additional nutritional load when the post-weaning environment was cafeteria diet and therefore developed glucose intolerance.

The evidence of pancreatic insufficiency was more remarkable in the offspring of the rats that were fed cafeteria diet during lactation. The development of pancreas is vulnerable to oxidative stress, which can result in premature pancreatic failure in later life *(Freeman, 2009).* Overnourishment of pups during lactation has been reported as a factor that can induce glucose intolerance *(Patterson et al., 2010).* It was shown that rearing rats in small litters during lactation (4 pups per litter) doubled body fat content and

plasma leptin concentration and led to hyperinsulinemia *(Schmidt et al., 2001).* In the current study litter size was standardized, therefore, the pancreatic insufficiency observed in the groups that were fed cafeteria diet during suckling may not be a result of overnourishment during early lactation. To fully exclude this possibility it would be necessary to sample milk and determine whether cafeteria diet altered composition, resulting in over-nutrition. The growth profiles of the pups suckled by cafeteria fed mothers would suggest that this is not the case. However, towards the final week of lactation these offspring were observed to consume cafeteria diet foods as well as their mothers. For this reason, in the light of observed outcomes, analyzing pancreatic structure and function at different points of the current study design, especially during early and late lactation becomes essential for future work.

Muscle tissues have been shown to be influenced by maternal high fat diets in several studies *(Bayol et al., 2005, Bayol et al.,2009, Samuelsson et al.,2008).* Importantly, in studies where greater adiposity was noted, elevated body fat percentage was shown to be more likely as a result of reduced lean mass than an increase in fat mass *per se (Buckley et al., 2005).* Furthermore, increased intramuscular deposition of fat could be thought of as a marker of systemic insulin resistance *(White et al., 2009).* Down-regulation of GLUT 4 and insulin receptor in muscle limits capacity for skeletal muscle glucose uptake impacting significantly on glucose disposal and tolerance. Therefore,

this is an area of interest for future studies of offspring from high fat diet fed mothers.

Reference was made, earlier in this chapter, to the thrifty phenotype hypothesis. Gluckman and Hanson revisited this hypothesis and generated the predictive adaptive response hypothesis to try to explain how early life influences a wider range of disease states. According to the predictive adaptive response hypothesis, the fetus makes an adaptation in the early postnatal development period based on the predicted postnatal environment. Disease manifests where a mismatch occurs between the predicted and actual environment *(Gluckman and Hanson, 2006).* Indeed, in our trials interactions between maternal obesity and cafeteria diet only during pregnancy, indicated that the offspring from these pregnancies had the most disturbed glucose homeostasis and body composition as a result of the greater mismatch between pre-gestational and pregnancy diets. Previously it was shown that rats fed a high carbohydrate diet exhibited extensive adaptations at molecular, cellular and biochemical levels in pancreatic islets *(Srinivasan et al., 2000).* Therefore, our findings may be explained by the distinct switches in the diets during pre-natal life as the predicted environment was altered. On the other hand, glucose intolerance in these animals was observed only when the post-weaning environment was cafeteria diet so this may suggest the adverse effects of a match rather than a mis-match between the pre-natal and postnatal nutritional environments. For

this reason, the data observed in this study may suggest a classic thrifty phenotype response.

The nature of the insult associated with cafeteria feeding which may mediate programming effects is unclear. In addition to the high-fat, low-protein nature of cafeteria diet, high salt and sugar intake may also have considerable effects on the development of the fetus. The individual or combined effects of these manipulations has been shown in several studies *(Ding et al., 2010, Sandberg et al., 1994, Oliveros et al., 1997, Kulthinee et al., 2010, Gray and Gardner, unpublished data).*

Sex specific effects of fetal programming have been shown in several studies (McMullen and Langley-Evans, 2005, Choi et al., 2007). In the present study, male offspring of cafeteria diet fed animals were found to be more vulnerable than female offspring in terms of programmed glucose intolerance and changes in gene expression. Other studies have shown males to be more susceptible to programming of hypertension and insulin resistance, with female offspring exposed to both maternal low protein and high fat diets being relatively resistant (*Sugden and Holness, 2002, Woods et al., 2005, Nivoit et al., 2009).* The causative factor underlying this observation has been attributed to the lack of estrogen in males. Estrogen may act as a pro-survival factor in females, by reducing the adverse effects of suboptimal maternal nutrition *(Ojeda et al., 2007).* It was shown that offspring of protein restricted animals developed glucose intolerance in both genders with similar mechanisms but that detrimental change simply occurred later in female

offspring *(Fernandez-Twin et al., 2005).* These studies highlight the sex- and insult-specific nature of developmental processes. In humans, gender-specific genes affecting insulin sensitivity were proposed to be responsible for the gender difference in birth weight *(Wilkin and Murphy, 2007).* The mechanisms by which sex specific pathways, are influenced by early programming have not been clearly elucidated but such findings illustrate the requirement for future research to determine the associated mechanisms.

The data presented in this study identified important outcomes to shape the design of future studies. It was primarily shown that the impact of maternal over-nutrition depended very strongly upon when the insult was imposed. Without the complex design this would not have been apparent. When the early life cafeteria diet treatment was combined with over-nutrition in the lactation and post-weaning periods, offspring exhibited glucose intolerance. Moreover, the mechanistic pathway leading to glucose intolerance was found to be dependent upon the dietary treatment during lactation, since cafeteria diet during lactation appeared to induce pancreatic insufficiency. In order to evaluate these mechanisms, future studies which give further understanding of the specific insults, such as high fat, high sugar or overnourishment during these specific periods of life will be of great interest. Assessment of pancreas and muscle tissues as well as plasma glucose and insulin concentrations at birth, during early and late lactation may produce a clearer picture of the mechanisms responsible. In addition to this, assessment of all the components of the insulin signalling pathway, at the

level of protein and RNA expression, will offer a broader understanding of the link between epigenetic and environmental factors. Animal studies can provide great understanding of the mechanisms by which these dietary manipulations exert lasting effects. Therefore, assessing the offspring that were produced in the current study design at an older age is crucial.

In conclusion, this study has developed a robust model for the evaluation of the independent effects of over-feeding and maternal obesity. The preliminary data showed interesting effects of maternal obesity, induced by cafeteria feeding in the pre-gestational period, on fetal and placental growth. These effects differed to the effects of cafeteria feeding during the pregnancy period alone. Data from our main trials generated results which contrasted with published literature. However, glucose intolerance due to the maternal cafeteria diet was shown to be developed regardless of the postnatal adiposity and food intake, but dependent on the postnatal nutritional environment. This may suggest the importance of the interactions between epigenetic mechanisms and the environmental factors.

Obesity in pregnancy carries not only maternal health risks but also increased risk for pregnancy outcome, the future health of the child and the health of the next generations *(Villamor and Cnattingius, 2006, Taylor and Poston, 2007).* If the findings of the current study and wider experimental literature apply to human metabolism and reproductive health, then the fetal programming hypothesis is of major public health importance. Given the current increase in Western diet-induced childhood obesity, maternal obesity

will be a major issue over the next 50 years. The long-term aim must be to reduce pre-pregnancy obesity and increase public awareness of the importance of a healthy diet before and during pregnancy. Governmental programmes and guidelines should be prepared to assist women to obtain adequate and optimal nutrition during these crucial periods of life. More robust and more prominently promoted recommendations to women about appropriate caloric intake, exercise and education about childhood nutrition and breast feeding should be developed. Moreover, effective management of gestational diabetes, maternal hyperglycaemia and hyperinsulinemia before pregnancy, during pregnancy and lactation may contribute to the prevention of adult diseases later in life.

7.0 APPENDIX

Table 7. 1 Nutritional values of cafeteria diet foods (Data obtained directly from manufacturers' information)

Food	Energy (Kcal/100g)	Protein (g/100g)	Fat (g/100g)	Saturated fat (g/100g)	Carbohydrate (g/100g)	Sugar (g/100g)	Sodium (g/100g)	Fibre (g/100g)
Rich shorties biscuits	500	6.1	23.2	10.6	65.6	23.6	0.3	0.3
Ready salted potato crisps	535	5.2	34.6	3.2	50.7	0.5	0.6	4.1
Vinegar salt potato crisps	525	5.2	33.1	3.1	51	0.5	1.0	3.9
Prawn cocktail potato crisps	525	5.3	32.8	3.0	52.1	1.9	0.6	4.0
Smokey bacon potato crisps	520	5.6	31.1	3.1	53.5	3.6	0.6	4.0
Cheese onion potato crisps	530	5.8	33.2	3.1	51.6	2.4	0.6	4.4
Fruit nut chocolate	490	8.3	25.9	14.6	55.8	55.2	0.08	1.4
Mars bar	446	4.1	17.4	9.8	68.3	59	0.17	1.2
Roasted and salted peanuts	600	25.2	51.5	8.9	8.0	4.5	0.3	10.1
Golden syrup cake	355	3.4	11.0	3.4	60.4	39.1	0.4	1.4
Pork pie	367	9	25.3	10.7	25.7	3.1	0.6	2.7
Cocktail sausages	275	12.7	19.5	7.1	12.0	1.2	0.6	0.5
Liver and bacon pate	285	13.5	24.0	9.0	3.2	1.0	0.7	2.1
Cheese	410	25.0	34.4	21.7	0.1	0.1	0.7	0
Jam	260	0.3	0.1	0	64	60.4	0	0

Table 7. 2 Formulation of B&K Universal standard chow diet

Ingredients	Composition
Crude oil (%)	4.73
Crude protein (%)	18.68
Crude fibre (%)	3.48
Ash (%)	5.38
NFE (%)	59.73
Digestible Crude Oil (%)	3.99
Digestible Crude Protein (%)	16.53
Gross Energy (MJ/Kg)	16.39
Digestible energy (MJ/Kg)	14.00
Vitamin A (iu/Kg)	14609.40
Vitamin D_3 (iu/Kg)	1504.10
Vitamin E (mg/Kg)	101.90
Thiamin (mg/Kg)	13.50
Riboflavin (mg/Kg)	11.80
Pyridoxine (mg/Kg)	14.40
Vitamin B_{12} (µg/Kg)	31.50
Vitamin K (mg/Kg)	16.00
Folic acid (mg/Kg)	2.80
Nicotinic acid (mg/Kg)	71.30
Pantothenic Acid (mg/Kg)	25.50
Choline (mg/Kg)	1390.20
Inositol (mg/Kg)	1714.00
Biotin (µg/Kg)	357.50
Calcium (%)	0.73
Total Phosphorus (%)	0.70
Magnesium (%)	0.18
Sodium (%)	0.22
Chloride (%)	0.44
Potassium (%)	0.69
Iron (mg/Kg)	87.00
Copper (mg/Kg)	18.30
Manganese (mg/Kg)	86.90
Zinc (mg/Kg)	92.50
Cobalt (µg/Kg)	456.80

Table 7.2 Formulation of B&K Universal standard chow diet (continued)

Iodine (µg/Kg)	1840.8
Selenium (µg/Kg)	391.80
Lysine (%)	1.10
Methionine (%)	0.33
Linoleic Acid (%)	2.04
Linolenic Acid (%)	0.18

Data obtained directly from B&K Universal Limited

Table 7. 3 Formulation of Teklad Global 18 % Protein Rodent Diet

Ingredients	Composition
Crude protein (%)	18.6
Fat (ether extract) (%)	6.2
Carbohydrate (available) (%)	44.2
Crude fibre (%)	3.5
Ash (%)	5.3
Energy (Kcal/g), (Kj/g)	3.1, 13.0
Calcium (%)	1.0
Phosphorus (%)	0.7
Sodium (%)	0.2
Potassium (%)	0.6
Chloride (%)	0.4
Magnesium (%)	0.2
Zinc (mg/kg)	70.0
Manganese (mg/kg)	100.0
Copper (mg/kg)	15.0
Iodine (mg/kg)	6.0
Iron (mg/kg)	200.0
Selenium (mg/kg)	0.23
Vitamin A (IU/g)	15.0
Vitamin D_3 (IU/g)	1.5
Vitamin E (IU/g)	110.0
Vitamin K_3 (mg/kg)	50.0
Thiamin (mg/kg)	17.0
Riboflavin (mg/kg)	15.0
Niacin (mg/kg)	70.0

Table 7.3 Formulation of Teklad Global 18% Protein Rodent Diet (continued)

Ingredients	Composition
Pyridoxine (mg/kg)	18.0
Panthotenic acid (mg/kg)	33.0
Vitamin B_{12} (mg/kg)	0.08
Biotin (mg/kg)	0.40
Folate (mg/kg)	4.0
Choline (mg/kg)	1200
C16:0 Palmitic Acid (%)	0.7
C18:0 Stearic Acid (%)	0.2
C18:1w9 Oleic Acid (%)	1.2
C18:3w6 Linolenic Acid (%)	0.3
C18:2w6 Linoleic Acid (%)	3.1
Total Saturated Acids	0.9
Total Monounsaturated Acids	1.3
Total Polyunsaturated Acids	3.4

Data obtained directly from Teklad Diets, UK

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