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Mechanisms underlying developmental induction of energy homeostasis and cardiovascular function in a mouse model: Impact of pre- and post-natal nutritional mismatch

By

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Abstract

Human and animal studies suggest that adulthood obesity may have its origins during early development. Maternal undernutrition during pregnancy has been shown to result in the promotion of increased fat deposition, obesity and associated metabolic and cardiovascular complications such as insulin resistance and hypertension. Little is known however, about how the type or extent of maternal undernutrition during pregnancy impacts upon the metabolic and cardiovascular phenotype of adult offspring. Also, the effects of extending maternal undernutrition to include the lactation period, on the metabolic and cardiovascular phenotype remain to be elucidated. In adult mouse offspring from dams that were either protein- or calorie-restricted during pregnancy and weaned onto a high-fat diet, we determined the mRNA expression levels of genes such as neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) in the hypothalamus, known to be involved in regulating appetite and the β-3 adrenergic receptor and uncoupling protein 1 (UCP1) in interscapulary brown adipose tissue, which are involved in the regulation of energy expenditure. We also determined the expression levels of these genes in adult mouse offspring from dams that were protein-restricted during pregnancy and lactation and fed a high-fat postweaning diet. In addition to analysis of gene expression we examined the effects of the aforementioned dietary paradigms on body weight trajectory, food intake, systolic blood pressure, blood glucose levels, energy expenditure and adiposity.

Pregnant MF1 mice were fed either normal diet (C, 18% casein), protein-restricted diet throughout pregnancy or pregnancy and lactation (PR, 9% casein), or received a 50% global calorie restriction during pregnancy. Weaned offspring were fed to adulthood a high-fat (HF; 45% kcal fat) or standard chow (C, 21% kcal fat).

Male HF-fed offspring from dams that were protein-restricted during pregnancy exhibited a 30% reduction in food-intake vs. male HF-fed offspring from normally-fed dams ($p<0.001$), while their body weight trajectory showed no changes. This effect was not evident if maternal protein-restriction was extended to include lactation wherein male HF-fed offspring from protein-restricted dams exhibited reduced body weight trajectory ($p<0.001$) with no changes in food intake. Despite this, HF-fed male offspring from dams that were protein-restricted during pregnancy and lactation had higher levels of adiposity, lower levels of energy expenditure and exhibited greater intramuscular fat deposition compared to male HF-fed offspring from normally-fed dams. There were no such changes in HF-fed offspring from dams that were protein-restricted during pregnancy alone. All HF-fed male offspring from protein-restricted dams, regardless of window of exposure to maternal undernutrition had reduced levels of UCP1 compared to their HF-fed counterparts from normally-fed dams ($p<0.05$). Male HF-fed offspring from dams that received a 50% calorie-restriction during pregnancy exhibited reduced energy expenditure (0.001), greater adiposity ($p<0.01$) and elevated blood glucose levels (0.05) compared to HF-fed male offspring from normally-fed dams. In all male and female offspring, maternal undernutrition followed by postweaning overnutrition led to a rise in systolic blood pressure over and above that which observed in HF-fed offspring from normally-fed dams ($p<0.05$).

Our results suggest that the metabolic phenotype of offspring is dependent on sex, type of maternal undernutrition during development and well as window of exposure to maternal undernutrition, whereas the cardiovascular phenotype is not. The most significant metabolic and cardiovascular changes are observed in HF-fed offspring from undernourished dams.

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Abbreviations

αMSH	Alpha melanocortin stimulating hormone
11βHSD	11β-hydroxysteroid dehydrogenase
5-HT1B	Hippocampal cyanopindolol to seratonin receptor 5
AGRP	Agouti-related peptide
ARC	Arcuate nucleus
BAT	Brown adipose tissue
CART	Cocaine and amphetamine-regulated transcript
CCK	Cholecystokinin
CRD	Cytokine receptor domain
CRP	C-reactive protein
DMN	Dorsomedial nucleus
Fn3	Fibronectin III
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotrophin releasing hormone
HF	High-fat
iBAT	Interscapulary brown adipose tissue
Ig	Immunoglobulin domain
IUGR	Intra-uterine growth-restricted
LHA	Lateral hypothalamic area
MC-R	Melanocortin receptors
NPY	Neuropeptide-y
NPY1-R	Neuropeptide Y receptor 1
NPY5-R	Neuropeptide Y receptor 5
ObRa	Leptin receptor (short form)
ObRb	Leptin receptor (long form)
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PR	Protein-restricted
PVN	Paraventricular nucleus
RMR	Resting metabolic rate
UCP1	Uncoupling protein 1
UN	Undernourished

Chapter 1

Introduction

1.1. Obesity epidemic

Obesity is a threat of epidemic proportions. In the UK, a prediction that one in three adults will be classed as obese within three years presents a major concern, not only for general health but also for the economy. It is predicted that by the year 2050, the total cost of combating obesity to the UK economy will be in the region of £30-40 billion [1]. The World Health Organization has established a scale system for the diagnosis of obesity based upon body-weight measurements and the subsequent mortality from diabetes, cardiovascular disease and stroke in developed countries. Body Mass Index (BMI) is used to denote body weight status, taking height into account. A BMI of greater than or equal to 25 is classed as overweight, with the first scale of obesity represented as a BMI of between 30 and 34.99. A BMI of between 35 and 39.99 is classed as the second scale of obesity, while a BMI of 40 and greater is the final obesity scale and is termed 'morbidly obese' and represents a critical value of health status [2].

Obesity is associated with a number of clinical manifestations collectively termed the metabolic syndrome. They include a clustering of various metabolic abnormalities common to obese patients, such as insulin resistance, glucose intolerance, dyslipidaemia and hypertension, and are risk factors for coronary heart disease and other cardiovascular complications [3]. Given that such factors could predict mortality from cardiovascular disease, a clear link between obesity and coronary heart disease has been established [3].

1.2. Is risk of obesity induced during early development?

The increased prevalence of obesity (within one generation) has prompted a shift in attitudes from a genetic cause of obesity to the acknowledgement of the influences of the environment and epigenetic mechanisms [4, 5]. It appears that obesity is multi-factorial, linking genetic, environmental and epigenetic components (Figure 1). In recent years, much work has been carried out to establish a link between genetic and environmental

factors to bring about obesity. It is widely considered that environmental exposures *in utero* and early postnatal life, such as alterations in maternal diet or exposure to maternal glucocorticoids, greatly contribute to the obesity epidemic [6-8]. These *in utero* factors could provide the means and opportunity for gene-environment interactions to bring about obesity in adult life.

1.3. Introduction to the Fetal Origins of Adult Diseases hypothesis

With obesity expected to grow in prevalence in the developing world, the identification of an underlying cause of obesity is of paramount importance. Both twin and adoption studies have implicated genetic and environmental factors respectively in the development of obesity [10, 11]. The Fetal Origins of Disease hypothesis suggests that the intra-uterine environment exerts a powerful influence over the subsequent development of obesity and associated metabolic disorders [12].

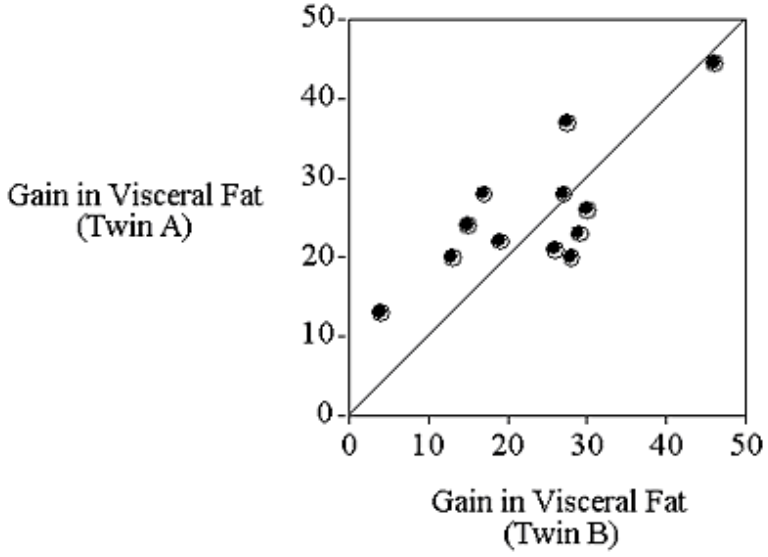


Figure 1. The effects of excess caloric intake on fat weight gain. Each point represents one pair of twins (A and B). The closer the points are to the diagonal line, the more similar the twins are to each other. The findings show the large variation between twin pairs and the little variation within twin pairs, demonstrating the strong influence of genes on resistance to diet-induced obesity. Very rarely however, do the points intersect the diagonal line however, meaning than environmental factors must play a role too. Taken from Stunkard et al. [10].

Epidemiological studies have been central to the fetal origins hypothesis, identifying clear associations between low birth weight and adult cardiovascular disease (figure 2) [13]. The Hertfordshire cohort study, designed to analyze the impact of the *in utero* environment on later disease risk in men and women born in the county of Hertfordshire between 1911 and 1939, confirmed the correlation between low birth weight and mortality due to heart disease [14]. A study analysing pathophysiological outcomes in pregnancy from the Dutch winter famine cohort, a group of three hundred thousand 19 year old men whose mother's were exposed to famine in the first trimester of pregnancy in 1944-45, established an increased risk of obesity [15]. This occurred in 1944 following a combination of a blockade of all food transports to the Netherlands by the retreating Germans and an unusually cold winter (the canals froze becoming impassable by barges) that led to food rations dropping to under 1000 calories per day [15]. Animal studies have since largely supported the initial observations made in humans with respect to the fetal origins of disease hypothesis. An animal model of maternal undernutrition whereby food intake was restricted by 50% throughout pregnancy in rats has shown there is a propensity for excessive fat accretion in the offspring, and consequent insulin-resistance [16].

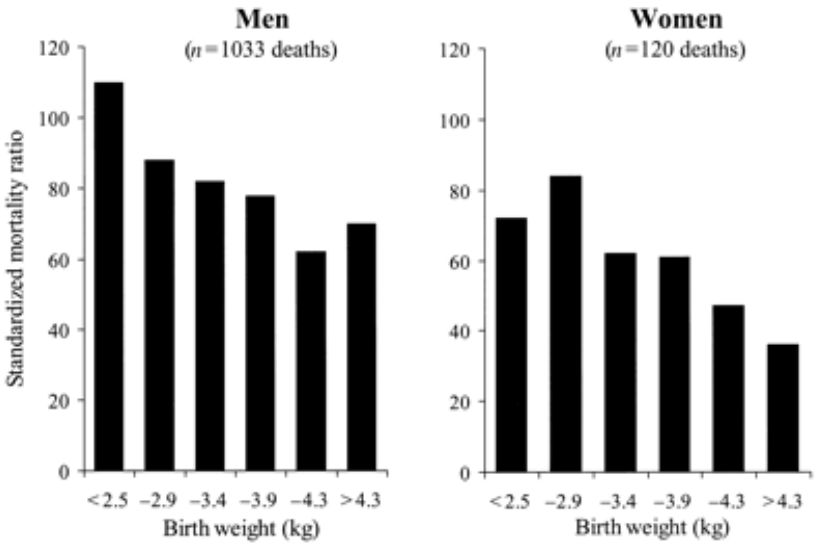


Figure 2. Coronary heart disease death rates, expressed as standardized mortality ratios, in 10141 men and 5585 women born in Hertfordshire, United Kingdom, from 1911 to 1930, according to birth weight. Taken from Godfrey and Barker [10].

Studies in animals have been widely used in research to illustrate the consequences of inadequate maternal nutrition to adult health. In the early to mid-20th century, owing to rationing and food shortages created by wars and prolonged economic recessions, maternal undernutrition during pregnancy was commonplace in developed countries. Inadequate nutrition, however, is no longer the problem it once was, as there is now greater economic prosperity, more nutritional awareness and higher level of education. In fact, the quandary that now occupies health-care professionals is a diet of abundance, one that promotes over-indulgence and is confounded by 'thrifty' genes or those that once provided protection during nutritional scarcity by promoting energy storage, but now exacerbate the problem posed by overnutrition [17]. Even in the developing world, industrialisation and improved economic prosperity, notably in India, China and parts of South America and Eastern Europe has led to a nutritional environment that is by no means dissimilar to that which is evident in the UK and US.

Overnutrition *in utero* is also not without its consequences. In a study of Danish military conscripts, BMI at ages 18-26 increased monotonically with birth weight [18]. Thus, a U-shaped curve (Figure 3), as explained by Yajnik [19] is commonly used to describe the relationship between birth weight and adult disease risk. This stems from the occurrence of increased disease risk following either nutritional excess or inadequacy.

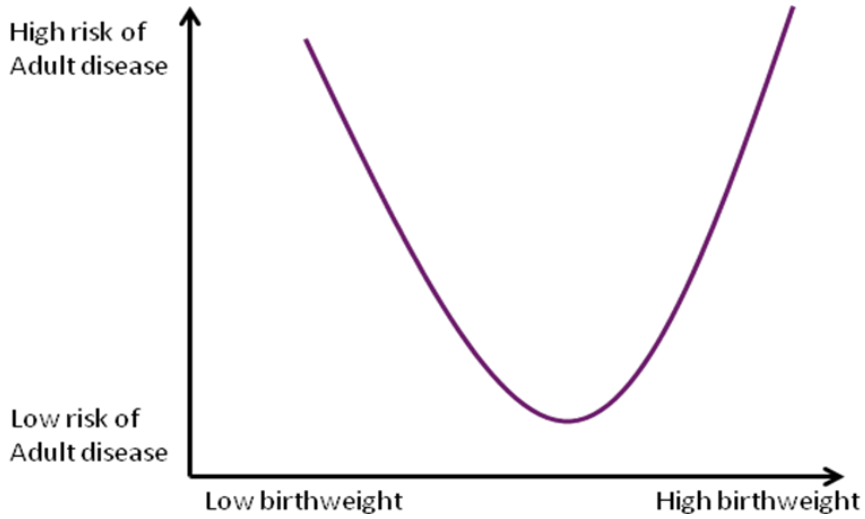


Figure 3. The U-shaped trend between birthweight and risk of disease in adulthood. Taken from Yajnik [19].

1.4. Developmental programming

Programming is the word that has been coined to explain the correlation between low birthweight and coronary heart disease in later life [12]. The mechanisms of programming are thought to occur during developmental stages of physiological 'plasticity', during which certain hormonal, neuronal and cellular mechanisms have a period in which to 'sensitise' to maternal nutrition. Exposure to an adverse intra-uterine nutritional environment during these critical windows may permanently alter the physiology, programming a new set of physiological set-points that may be responsible for the development of adult obesity and cardiovascular and metabolic disease.

1.4.1 The Thrifty Phenotype hypothesis

The thrifty phenotype hypothesis as explained by Hales and Barker [9] describes how developmental programming might occur in response to poor fetal nutrition, leading to permanent changes in glucose and insulin metabolism. The programming events have been postulated to enable survival in a postnatal environment based on maternal nutritional cues. The 1944 Dutch winter famine supports the thrifty phenotype hypothesis by suggesting that adult offspring from mothers who were malnourished during pregnancy have an increased rate of fat storage, hence a predisposition to weight gain and obesity [15]. The only shortcoming of this theory is that it cannot explain instances of fetal programming that occur following overnutrition, or why in some deprived countries *in utero* undernutrition is not associated with programming of metabolic disease in adulthood. Gluckman and Hanson [20] have proposed an alternative theory which has been termed the predictive-adaptive response theory.

1.4.2. The Predictive-Adaptive Response theory

The predictive-adaptive response theory proposes that the fetus makes physiological adaptations *in utero* that are appropriate for the predicted postnatal nutritional status [20]. Such developmental alterations seem to confer advantages on the fetus, but only when the predicted postnatal environment is similar to what has been predicted *in utero.* These metabolic adaptations may not necessarily provide any benefit *in utero.* Evidence certainly suggests that poor intra-uterine nutrition promotes altered physiology that favours energy storage [21]. It has also been proposed that metabolic changes that result from fetal malnutrition, such as insulin resistance are programmed events that enable the redistribution of nutrient supply in favour of vital organs but to the detriment of muscle metabolism [22]. This then results in some form of compensatory adaptation of the pancreas to maintain glucose homeostasis, culminating in pancreatic beta cell exhaustion and subsequent development of type II diabetes [23]. Thus if a disparity between the pre- and postnatal environment exists, such as when fetal malnutrition is preceded by an energy-rich postnatal environment, the predicted postnatal nutritional environment is likely to be incorrect. This gives rise to inappropriate adaptive responses leading to the manifestation of disease. On the other hand if the predicted nutritional environment is correct, then disease is most unlikely to be manifested in the offspring. This may help to explain why in parts of Africa, fetal malnutrition is not associated with diabetes and obesity [24],

Gluckman and Hanson have alluded to the evolutionary basis of the predictive adaptive response in their book ‘The Fetal Matrix’ [20]. It explores the possibility that evolution has allowed for programming during vital stages of fetal growth. The authors suggest that the increased propensity of the malnourished fetus to store fat following birth is a survival mechanism that has evolutionary origins. Thus, the predictive adaptive response capability that allows programming has been preserved by evolution. This greatly contributes to the

ability to adapt to the external environment, even before the fetus has been exposed to it. Such adaptive abilities have allowed mammals, and specifically humans, to survive in constantly changing environments.

1.5. Animal models of programming

Human studies have provided epidemiological evidence for the notion of developmental programming, while animal models have since been used to examine the mechanisms involved in this phenomenon. Such models allow the detailed study of events that give rise to developmental programming and its consequences.

1.5.1. Fetal undernutrition

A number of animal models have been used to replicate the human scenario of fetal malnutrition. The two most commonly used animal models are of global undernutrition by restricting total food intake and the restriction of specific nutrients such as protein [25, 26]. Protein is one of the most important macronutrients and plays an important part in various physiological processes. Thus reducing the amount of protein in the maternal diet has been widely used in studies investigating developmental programming. An advantage of using this model of maternal dietary manipulation as opposed to a more extreme maternal nutritional insult, such as global undernutrition, is that offspring viability and litter-size remain unaltered [27,28]. Nevertheless, offspring of protein-restricted dams have reduced birth weights compared to their control-fed counterparts. This low body weight is maintained throughout life [25]. Moreover these offspring, particularly males, tend to develop glucose intolerance and diabetes with age [29].

It appears that following exposure to a maternal protein-restricted nutritional environment, the growth of the brain is unaffected and is proportionally larger than those found in offspring from normally fed dams [30]. This ‘brain sparing’ effect is largely believed to be

an adaptive mechanism protecting this vital organ. A study has shown decreased brain metabolism, hence lower energy requirements of this organ, in rat offspring exposed to maternal protein-restriction *in utero* [31]. As shown in Figure 4A, oxygen consumption is significantly reduced in the low-protein group at birth, a difference which is still evident the day after birth (Figure 4B). In sheep, redistribution of blood flow in favour of the brain during protein restriction has been suggested to contribute to protecting brain development, despite the adverse nutritional insult [32]. Vascularisation of the developing brain itself has been shown to be reduced, reflecting the reduced energy demands [33].

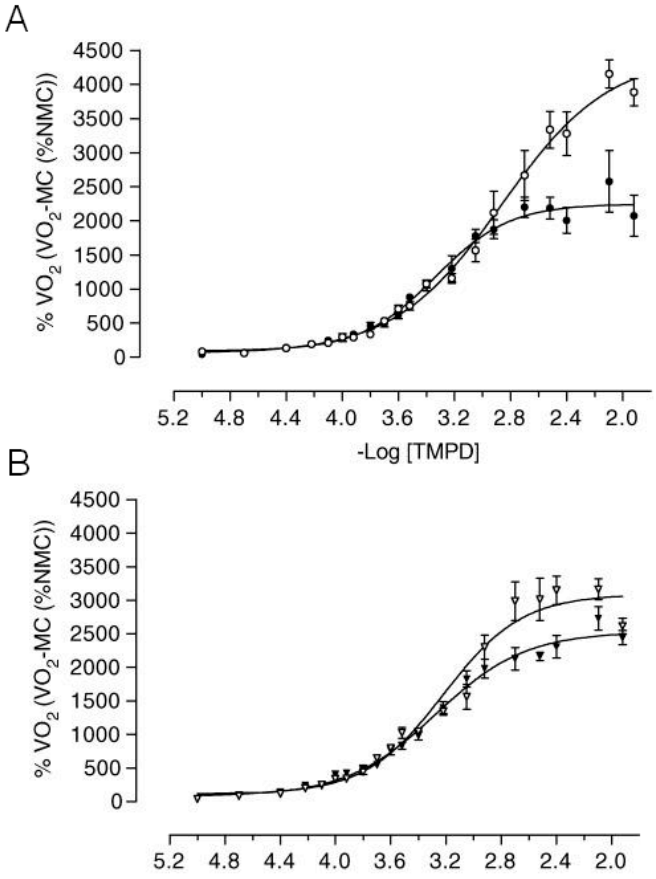


Figure 4. The effect of maternal low protein diet on oxygen consumption before and after birth. Values are mean ± s.e.m. TMPD dose–response curves. A, the day before birth (E21) in control (◦, *n* = 13) and low protein (•, *n* = 7) groups; B, the day after birth (P1) in control (▲, *n* = 9) and low protein (▲, *n* = 7) groups. MC, myxothiazol control; NMC, non-myxothiazol control. Figure taken from Gallagher et al. [30].

While brain development in the fetus is maintained, other organs and organ systems are more negatively affected as a result of undernutrition during pregnancy. Glucose metabolism has been shown to be altered as a result of maternal protein restriction during pregnancy. Offspring of protein-restricted rat dams are found to have increased glucose sensitivity in the immediate postnatal period. However, impaired glucose tolerance begins to appear with age leading to the development of diabetes [33]. The precise mechanisms for this occurrence are difficult to ascertain given the plethora of hormones, enzymes, substrates and organs involved. However the pancreas and liver are prominently affected by maternal protein restriction during pregnancy. Certainly hepatic enzymes involved in glucose metabolism have reduced activity [34], while the effect of glucagon on glucose output is attenuated [35]. Pancreatic structure and function in the offspring have been shown to be reduced following a maternal protein-depleted diet during pregnancy, with beta-cell mass and vascularisation negatively affected [36] and islet cell function compromised [37].

1.5.2. Fetal overnutrition

As explained previously, there is a U-shaped trend in the relationship between birthweight and risk of adult disease, with high birthweight also leading to deleterious metabolic consequences. While this thesis will not focus on fetal overnutrition it is important to be aware of how maternal overnutrition during development might lead to obesity and cardiovascular consequences in later life. It is plausible that similar mechanisms of programming might affect both offspring from undernourished as well as overnourished dams. Caloric excess is rather difficult to replicate in an experimental environment, particularly when applied to rodents. Rats and mice in particular have very efficient means of regulating energy expenditure and food intake to guard against any deviation from a body weight set-point [38]. This can be negated somewhat by a highly palatable diet consisting of high levels of fat. Animal experiments of *in utero* overnutrition have

manipulated the nutritional composition of the mother's diet to mimic the 'Western' or 'cafeteria' diet which comprises a greater percentage of fat. Bayol and colleagues [39] adopted a highly palatable diet of processed foods containing large amounts of carbohydrates and fat which was offered to pregnant rats. Among the items offered to the rats were jam doughnuts, biscuits, and cheese. The cafeteria diet contained 19% fat compared with 3.27% in the chow diet. Offspring from mothers fed the cafeteria diet during pregnancy showed physiological abnormalities consistent with early indications of the metabolic syndrome and obesity. Such offspring have unusually high amounts of intra-muscular fat deposits and overall adiposity at maturity was significantly higher than age-matched controls.

In humans, one of the earliest reports of fetal dietary excess and subsequent development of obesity in later life was derived from studies undertaken in Pima Indians, a group of indigenous peoples living in an area consisting of what is now Central and Southern Arizona (USA) and Sonora (Mexico). The Pima Indians were found to have a grossly disproportionate prevalence of obesity and diabetes. A study in a population of Pima Indians aimed at elucidating the nature of the relationship between fetal nutrition, obesity and type II diabetes found that offspring exposed to maternal diabetes during pregnancy, had higher birthweights and BMI in adulthood [40]. Because maternal glucose is readily transferred across the placenta to the fetus while insulin is not, the fetus responds by producing excessive amounts of insulin, which not only acts as a growth hormone, but also serves to increase fuel storage. Thus, maternal diabetes has been argued to be an important contributor to fetal programming of obesity. Studies in humans addressing fetal overnutrition have also suggested an association between high birthweight and high BMI in later life [41, 42]. This thesis will not focus on maternal overnutrition during development, but will determine the effects of post weaning overnutrition following maternal undernutrition.

1.5.3. Post weaning overnutrition

A number of studies have demonstrated the ability of a diet high in fat to induce obesity and metabolic disease in rodents [43-45]. Rodents have efficient means of defending against body weight gain, by induction of thermogenesis in their interscapulary brown adipose tissue [46]. When presented with a high fat diet, mice have also been shown to initially reduce food intake, possibly through an elevation of plasma leptin levels [45]. Despite these defensive mechanisms, however, the high fat diet leads to increased adipose mass and weight gain over time [45]. This high fat diet-induced obesity has been demonstrated to lead to insulin resistance [47], type-2 diabetes [48-50] and hypertension in rodents [51].

1.5.4. Nutritional mismatch

With improved living standards and increased economic prosperity, changes in diet that accompany this transition in many parts of the developing world necessitate the need to develop a more appropriate nutritional model to represent the socio-economic transition that is characteristic of countries such as India, China and Brazil. The rapid economic expansion in these countries has presented some sort of dilemma. Women in these countries, who were once living in poverty, are now experiencing comparable prosperity and this is reflected by their having access to more foodstuffs. Unfortunately, these are mainly processed foodstuffs which are high in fat and carbohydrates. There is an obvious implication for children exposed prenatally to a nutritionally scarce environment, and are subsequently brought-up in a nutritional environment in which food is in plenty supply but at the same time is high in fat and carbohydrates. Based on the predictive-adaptive response theory, these children are at greater risk of developing metabolic and cardiovascular disease in later life.

Several animal studies have attempted to replicate this scenario under experimental conditions. A rat model was developed in which dams were undernourished during pregnancy while their offspring were exposed to a hypercaloric diet following weaning [26]. Mean birthweight of the offspring from the dams that were undernourished during pregnancy was found to be significantly lower than controls, which is in accordance with results obtained in the majority of studies addressing fetal undernutrition [52, 53]. A nutritional mismatch was then introduced by cross-fostering a group of offspring from dams that were undernourished during pregnancy with dams fed a normal diet. These cross-fostered offspring already exhibited exaggerated weight gain and increased adiposity. Increasing the degree of mismatch by feeding these offspring a hypercaloric diet from weaning resulted in increased weight gain and adiposity. This nutritional mismatch was shown to be responsible for the observed postnatal catch-up growth in these offspring.

'Catch-up growth' is used to describe the rapid nature of early postnatal weight-gain following fetal undernutrition [26]. It is thought to contribute to later diet-induced obesity in undernourished fetuses and has also been shown to be detrimental to longevity due to increased telomere shortening [26, 54]. Catch-up growth has been demonstrated in high fat-fed offspring from dams that were fed a protein-restricted diet during pregnancy [26]. Interestingly, in rodents that have undergone catch-up growth, the greatest difference in weight gain between these offspring and controls was in the initial stages of postnatal development. This indicates the importance of this period in determining later development of obesity [55, 56]. It is likely that prenatal undernutrition leads to programming of obesity which is further exacerbated by postnatal overnutrition. The degree of weight gain and adiposity induced by post weaning high-fat feeding alone is less than if preceded by prenatal undernutrition, [56], Thus, both the pre- and early post-natal nutritional environments can influence the development of obesity and catch-up growth. Furthermore, exposure to maternal undernutrition during pregnancy seems to program

physiological mechanisms that regulate food intake. In one study in rats (see Figure 5) postnatal hyperphagia was observed in offspring from dams that were undernourished during pregnancy [26]. Interestingly, in sheep, hyperphagia has also been shown to act in conjunction with increased energy efficiency (i.e. lower energy expenditure) to promote adipose tissue deposition during postnatal catch-up growth [40].

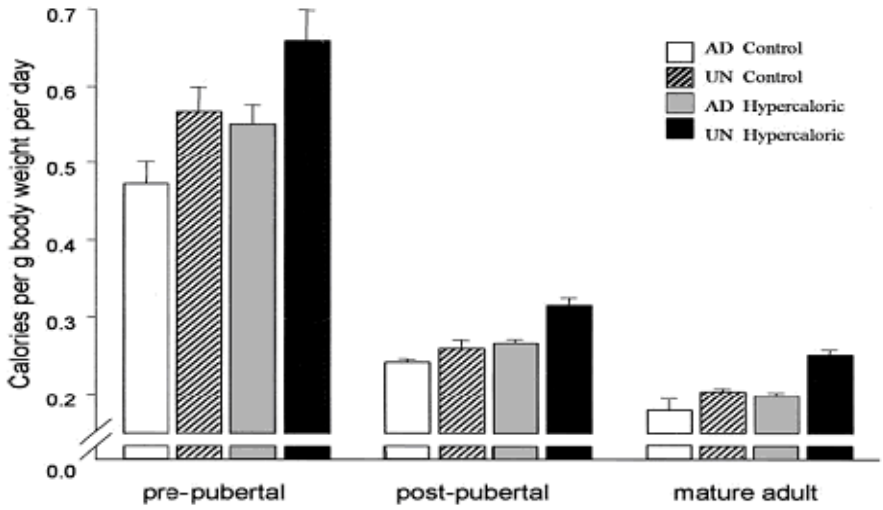


Figure 5. Caloric intake per day in offspring from *ad libitum*-fed (AD) and underfed (30% of AD diet, UN) mothers, during 3 distinct postnatal age periods (of ~3 wk each), fed either a control or a hypercaloric diet. Data taken from Vickers et al. [26].

1.6. Window of Exposure to maternal undernutrition

The early environment appears to play a significant role in the development of disease in later life, as shown in most animal and epidemiological studies. The thrifty phenotype hypothesis suggests that sub-optimal fetal nutrition permanently alters metabolic and cardiovascular function predisposing to disease in adulthood [12]. What remains to be elucidated is the extent to which window of exposure to maternal undernutrition influences the metabolic and cardiovascular phenotypic outcome. Current literature pertaining to window of exposure to maternal undernutrition is inconclusive. Some studies have suggested that the late gestation period is critical to the development of hypertension [57, 58]. This may be due to the fact that nephrogenesis begins in the last trimester of pregnancy in most mammals [59]. In terms of cardiovascular phenotype, other studies have

suggested that the postnatal period is also critical [60]. Accelerated postnatal catch-up growth during the lactation period in mouse offspring was shown to have a deleterious effect on their cardiovascular function [61]. Conversely, appropriate levels of nutrition during lactation in rats, was shown to have a positive effect on cardiovascular health of the offspring [62]. Moreover, adequate nutrition during lactation in rats seemed to prevent the deleterious effects of maternal undernutrition during pregnancy on nephron numbers and blood pressure in the offspring [63]. These findings suggest that both the prenatal period and early postnatal periods influence the susceptibility of offspring to disease. While nephrogenesis begins in late gestation, it is not complete until about two weeks after birth [60]. Therefore both maternal undernutrition during pregnancy and lactation may influence kidney development. A study in rats has attempted to determine the effects of undernutrition during pregnancy and lactation on growth trajectory and metabolism in offspring [64]. It showed that window of exposure was critical to growth, with offspring from dams fed a protein-restricted diet during pregnancy and lactation or exclusively during lactation exhibiting reduced growth trajectories compared to offspring from dams that were fed the protein-restricted diet during pregnancy alone [64]. The study in question suggests that the early postnatal period, has more influence over adult growth. The hypothalamic region of the brain, which regulates appetite and metabolism, is still developing at the time of birth, and the neuronal interconnections within this brain region are not fully established until 3-4 weeks after birth [65]. This may explain why the lactation period is important to growth trajectory of offspring.

1.7. Developmental programming of obesity

Obesity is a disorder of energy balance, whereby energy intake exceeds energy expenditure. A recent study in the UK has revealed that, not only is there a rapidly progressing prevalence of obesity amongst young people, but there is also a geographical pattern of obesity, that is strongly associated with regions of greater relative poverty [66].

Obesity is a major health problem due to its related life-threatening complications that manifest with increasing adiposity.

The pathophysiology of many diseases ascribed to developmental programming, such as coronary heart disease, hypertension, insulin resistance and dyslipidaemia, most likely involve prior development of obesity. Thus obesity is most likely to be one of the main phenotypic features of the aforementioned diseases as well as in some forms of cancer. The multifactoral nature of obesity, involving both genetic and environmental influences, must be treated accordingly. Thus, in order to better understand obesity, the focus should be on investigating the gene-environment interactions that causes it.

The Dutch winter famine study, amongst others, has demonstrated the role of developmental programming of obesity in later life [15]. This epidemiological study has shown that offspring from mothers who were malnourished during pregnancy gave birth to offspring that were predisposed to fat accretion in adulthood. The precise timing, however, of the programming events is difficult to establish, given that early postnatal nutrition has been implicated in the development of obesity in adulthood. Certainly, studies showing the unfavourable effects of bottle-feeding vs. breast-feeding in infants (see Figure 6) suggest the importance of the early postnatal environment [67].

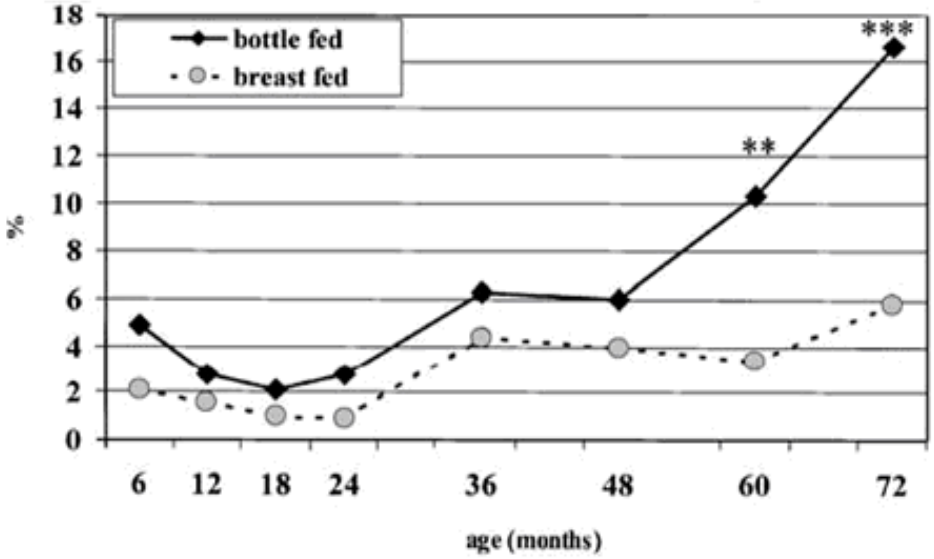


Figure 6. Prevalence of overweight, according to BMI. Proportion (%) of children exceeding the 90th percentile of the BMI reference values, depending on feeding mode in infancy. Data taken from Bergmann et al. [67].

Clearly, a U-shaped trend with regard to birthweight and later development of obesity and associated conditions exists, with either fetal undernutrition or overnutrition predisposing to increased adiposity and metabolic dysfunction. Mechanisms contributing to the development of obesity remain to be elucidated but recent studies have focussed on known biochemical pathways and genes involved with energy intake (appetite regulation) and energy expenditure (metabolic regulation).

1.7.1. Developmental programming of mechanisms regulating food intake

When considering mechanisms of fetal programming of obesity, attention commonly focuses on alterations in mechanisms that regulate food intake. This is based on numerous findings of altered food intake resulting from adverse intra-uterine nutritional challenges. One study in rats has observed altered food intake in offspring exposed to a high-fat diet *in utero* [68], while others have demonstrated hyperphagia or increased food intake in offspring from undernourished dams fed post-weaning a hypercaloric diet [26]. A more

recent study in mice has demonstrated that offspring from dams fed a high fat diet during pregnancy are prone to overeating in adulthood [8].

1.7.2. Neuroanatomy of the hypothalamus

The hypothalamus, located at the base of the brain is responsible for processing peripheral signals and initiating appropriate responses to regulate food intake and energy expenditure regulation. Figure 7 shows the key appetite regulating pathways within the hypothalamus. The arcuate nucleus (ARC) of the hypothalamus contains two groups of neurons based on their biological effects. The first group contains a population of neurons that co-express the neuropeptides agouti gene-related peptide (AGRP) and neuropeptide Y (NPY). The second group of neurons co-express pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) [69]. These neurons in turn project to second order neurons in other regions of the hypothalamus, notably the paraventricular nucleus (PVN), dorsomedial nucleus (DMH) and lateral hypothalamic area (LHA) [69]. Given that in rodents, the neuronal projections from the ARC to second order neurons are still in the process of development by the time of birth, the hypothalamus is thus regarded as a potential region of the brain where programming could occur [65].

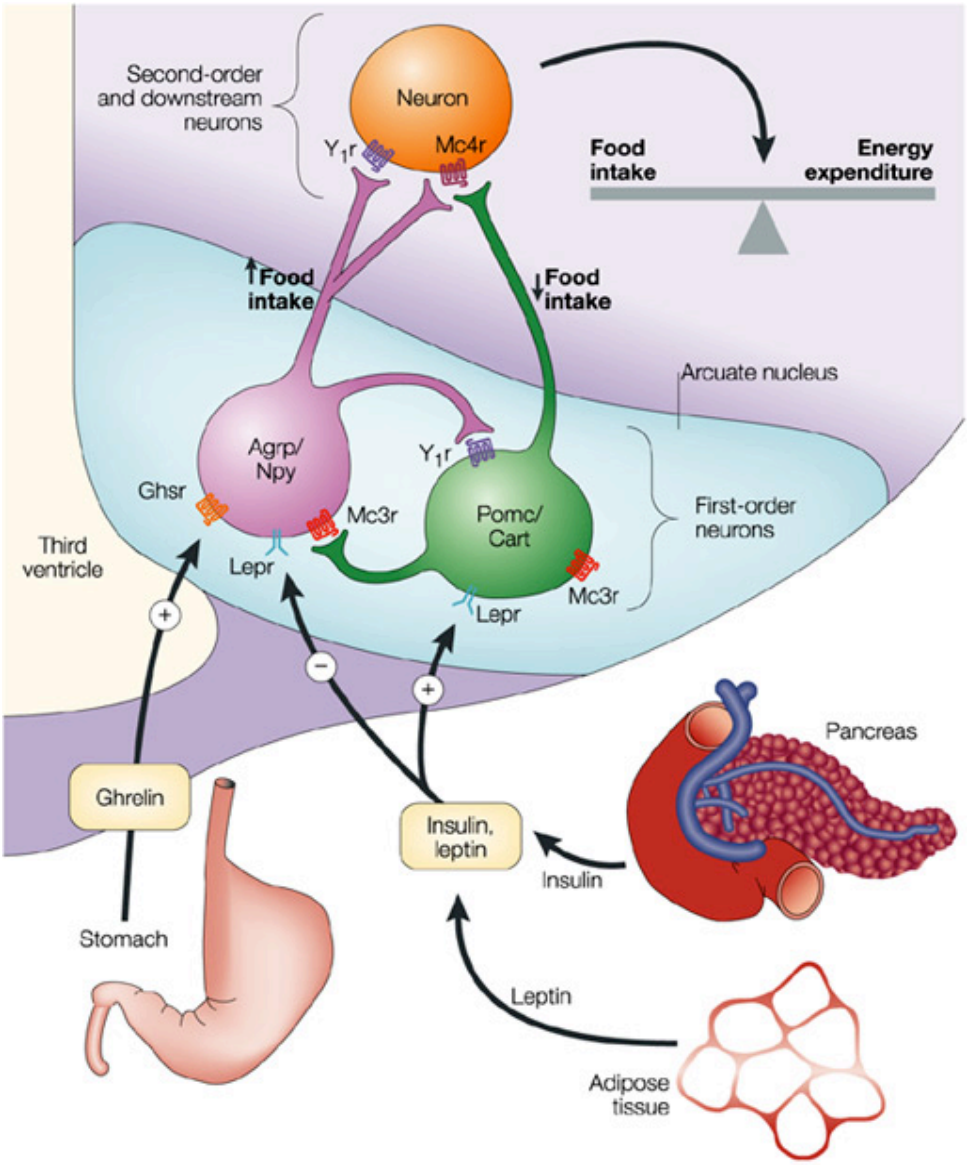


Figure 7. Appetite-regulating pathways within the hypothalamus. Lepr: Leptin receptor; GhSr: growth hormone secretagogue receptor,Mc3r: Melanocortin 3 receptor; Mc4r: Melanocortin 4 receptor; AgRP: Agouti related peptide; NPY: Neuropeptide-Y; Pomc: Pro-opiomelanocortin; Y_1r: NPY Y1 receptor. Figure taken from Barsh and Schwartz [70].

1.7.3. POMC

POMC expressing neurons are abundantly expressed in the ARC. POMC is the common precursor for a number of melanocortins including alpha melanocortin stimulating hormone (α-MSH), all of which exert their effects through the activation of the melanocortin receptors (MC-R), of which three are found in the brain, namely; MC4-R,

MC3-R and MC5-R [69]. Antagonism through selective blockade of the MC-R in the hypothalamus leads to hyperphagia and reduced metabolic rate, ultimately leading to the development of obesity [71]. Thus the actions of the melanocortins are concerned with prevention of weight gain by inhibition of feeding (anorexigenic effect) and increasing energy expenditure [71] as well as decreased insulin secretion and increased fasting plasma glucose levels [69]. Not surprisingly, a phenotype consistent with obesity and metabolic syndrome was described in studies on MC4-R 'knockout' mice [72], while an exogenous MC4-R selective antagonist was shown to produce a similar phenotype [73].

1.7.4. NPY

NPY is a potent promoter of feeding (producing an orexigenic effect), effectively counteracting the effects of the melanocortins [69]. Activation of NPY producing neurons by leptin leads to hyperphagia and inhibition of sympathetic nervous system activity to brown adipocytes (a target site of thermogenesis) leading to decreased energy expenditure. Obesity rapidly develops if central administration of NPY into the brain is maintained for several days, subsequently leading to insulin resistance [74]. NPY is markedly increased during fasting and is inhibited by leptin [70]. The major orexigenic actions of NPY have been demonstrated to occur via hypothalamic NPY receptors NPY1-R and NPY5-R [75]. As neuronal projections in regions of the hypothalamus involved in appetite are not fully established in rats until around 20 days after birth, an experiment was devised [76] in which the litter size was reduced, leading to gestational overnutrition. This resulted in the programming of the NPY response mechanism such that NPY inhibition was no longer observed at appropriate times, such as following a meal. The result is that the normal negative feedback of feeding by NPY fails to occur in overnourished pups which may be accompanied by decreased energy expenditure. A decrease in NPY has also been observed in fetal sheep exposed to nutrient restriction, further implicating NPY programming in models of obesity following fetal nutrient restriction [77].

1.7.5. Other hypothalamic neurotransmitters involved in appetite regulation

AGRP appears to exert its orexigenic effects through antagonistic action on the MC4-R [78]. Transgenic mice with AGRP overexpression became obese and insulin resistant [78], reflecting an identical phenotype as that observed in MC4-R knockout mice. An immunohistochemical study has also shown that AGRP neurons are highly co-localised with NPY neurons [69].

Galanin, a neurotransmitter consisting of 29 amino acids, is co-expressed in the hypothalamus with gonadotrophin releasing hormone (GnRH) neurons. Its diverse functions range from reproductive regulation and sexual behaviour to pain amelioration [79, 80]. Galanin is ubiquitously expressed, both within the CNS and periphery, particularly in the muscle. Galanin has also been shown to modulate feeding behaviour. Several studies have established the orexigenic effect of galanin exerted via its various receptor subtypes [81, 82, 83], an action that can be prevented by the administration of the galanin receptor antagonist Galantide [84]. Studies addressing programming of appetite in rats have revealed altered expression of galanin receptor subtype 2 in the hypothalamus following *in utero* exposure to maternal protein restriction [85].

1.7.6. Leptin and leptin receptors

Leptin, a hormone secreted by adipocytes is one of the major satiety signals, facilitating the termination of feeding to maintain body weight homeostasis by preventing overfeeding. Although produced peripherally mainly in adipose depots, leptin can exert its actions centrally within the hypothalamus via the leptin receptors that are abundantly expressed in this region of the brain [86]. The role of leptin in regulating food intake has been extensively researched following studies in rodents demonstrating leptin-deficiency resulting in hyperphagia and obesity [86]. Production of leptin is stimulated by compounds

involved in nutritional influx such as glucocorticoids and insulin [87]. Leptin activation of its receptor in the hypothalamus leads to decrease in food intake and increase in lipid mobilization and subsequent energy expenditure [88]. Increased adipose tissue is associated with increased circulating leptin, thus the hormone is a fairly reliable predictor of energy stores [89].

The leptin receptor exists in multiple forms due to alternative splicing of the leptin receptor gene [90], producing transcripts of different length. The two main isoforms are named the short and long forms, abbreviated to Ob-Ra and Ob-Rb, respectively. Ob-Rb has been shown to be the regulator of leptin's effects on appetite and metabolism through its actions on POMC- and NPY-producing neurons [88]. Figure 8 shows the various leptin receptor isoforms.

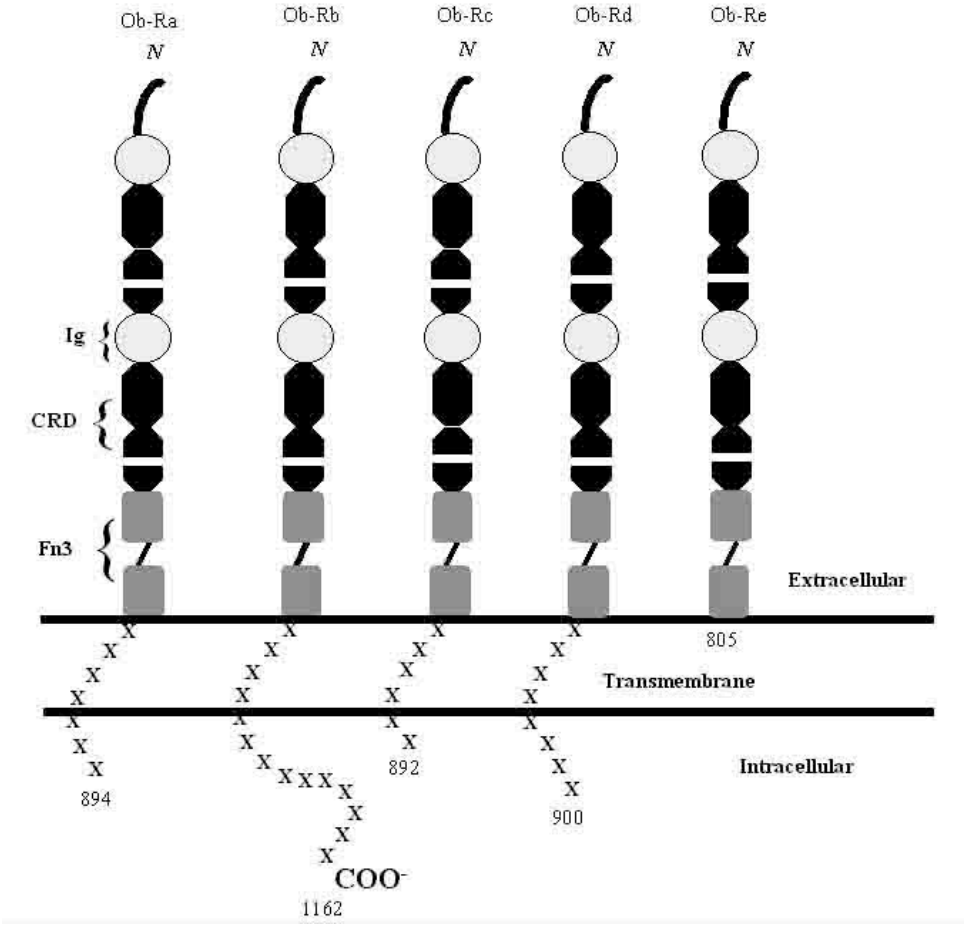


Figure 8. Leptin receptor isoforms showing their structural motifs. Ig: Immunoglobulin domain, CRD: Cytokine receptor domain, Fn3: Fibronectin III domain. Figure taken from Tartaglia [90].

Evidence of reduced circulating leptin levels have been observed in offspring that were malnourished during fetal development [91, 92, 93]. Decreased plasma leptin at birth in malnourished fetuses has been postulated to offer an adaptation to a predicted nutrient deprived postnatal environment. In one study, rapid postnatal catch-up growth was observed in previously malnourished offspring reaching a bodyweight which exceeded that of well-nourished control offspring [91]. By 3 weeks, the malnourished offspring exhibited elevated plasma leptin levels compared with controls, which increased further by 9 months of age. Hyperphagia was observed in the malnourished offspring despite the elevated leptin levels, suggesting leptin resistance. Also, resistance to the satiety stimulating actions of insulin following fetal malnutrition was found to be exacerbated by a postnatal hypercaloric diet, demonstrating the additive effects of postnatal overnutrition [26].

Studies in rats where litter size was reduced to encourage overfeeding demonstrated a considerable degree of insulin and leptin resistance, with elevated leptin levels having little effect on appetite suppression [94]. Increasing litter size had the opposite effect on insulin and leptin levels, and it was suggested that the hypothalamic NPY system may have been altered as a result of altered neuropeptide signalling in early postnatal life [95].

Central leptin action also occurs through the Ob-Rb present on subpopulations of neurons with the hypothalamus [90]. It then stimulates the melanocortin pathway, thus providing an alternative mechanism for insulin's effects on appetite and energy expenditure [90]. This was shown in a study where blockade of MC4-R, using an exogenous antagonist, completely abolish the central action of leptin on feeding and energy expenditure [96]. A lowering of leptin levels is associated with a proportionate decrease in POMC levels and subsequently that of α-MSH. Conversely, a rise in leptin levels has the opposite effect on POMC and α-MSH levels. Thus leptin appears to function in concert with the melanocortins via MC4-R, preventing hyperphagia and obesity.

A subpopulation of both NPY- and POMC-containing neurons expresses Ob-Rb [69]. The function of Ob-Rb on NPY-containing neurons, in contrast to that of POMC, is to inhibit NPY expression and thus counteract orexigenic effects. In light of a recent review [97], this inhibition of NPY is the favoured pathway of leptin actions, taking precedence over alternative modes of action. This may reflect the huge potency and strength of NPY in eliciting its appetite-stimulatory action.

The appetite regulatory system of the brain appears to be affected by the type of milk consumed by the offspring during the lactation period (i.e. breast vs. formula milk). The controversy surrounding breast-feeding vs. formula-feeding debate arises from data clearly showing that formula-fed infants are predisposed to developing obesity in adulthood [98].

Breast milk contains lower amounts of protein and fat than its manufactured counterpart, thus differential substrate exposure (due to the difference in the nutrient composition of the milk) is suggested to impact upon development of appetite regulatory systems [99]. The observation of elevated circulating leptin levels in breast-fed infants undoubtedly exerts an anti-obesity effect [100]. The role of leptin in the early postnatal environment in mice is unclear. Nevertheless there is emerging evidence for its possible involvement in neuronal maturation. While leptin exerts powerful appetite-suppressing effects in adult mice, it has no impact on appetite up to 10 days after birth. Only by 28 days of age are offspring responsive to leptin [101].

Nevertheless this first 28 days of life has been shown to be the critical period for the development of the neuronal circuitry involved in the regulation of appetite [102]. Thus, any changes in the *in utero* environment brought about by alteration in the maternal diet during pregnancy could therefore affect circulating leptin levels, which in turn may alter synaptic connections between neurons within the arcuate region of the hypothalamus involved in appetite regulation. This is therefore a critical period of plasticity.

Food intake is just one of two components of energy balance, the other being energy expenditure. If energy intake exceeds energy expenditure, weight gain occurs. On the other hand, weight loss occurs if energy expenditure exceeds energy intake. Thus, a perturbation in the mechanisms regulating energy expenditure might also lead to obesity.

1.8. Mechanisms underlying programming of energy expenditure

1.8.1. Regulation of energy expenditure

Energy expenditure has several components (see Figure 9). Obligatory energy expenditure is analogous to resting metabolic rate (RMR) and accounts for energy required for the fulfilment of cellular obligations. This value is fairly constant provided that body composition is maintained. RMR is increased with increased fat-free mass (muscle mass). Peripheral sites such as the thyroid, adrenals, muscle, fat, reproductive tissue and gastrointestinal organs represent the origin of afferent signals to the brain that determines metabolic status. Hormones and neurons are involved in the transmission of input to the brain, which it processes before initiating an appropriate response via efferent signals [103, 104]. Cholecystokinin (CCK) is one of the most influential afferent signalling hormones regulating energy expenditure. This gastrointestinal hormone is secreted after ingestion of a meal in response to distension of the gut wall, where it acts on the brain to inhibit appetite [105]. Physical activity energy expenditure refers to the portion of calories used during physical activity, while adaptive thermogenesis occurs in brown adipocytes in response to feeding and a decline in core body temperature [106].

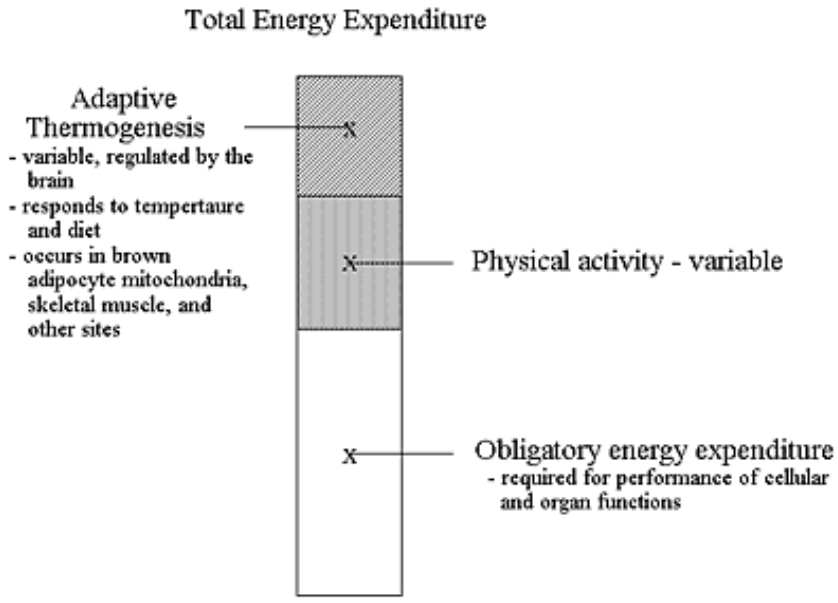


Figure 9. The three major components of energy expenditure. Taken from Lowell et al. [106]

There is a plethora of evidence for reduced energy expenditure-induced obesity in rodents. Studies have shown that the leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice not only demonstrate hyperphagia but also exhibit reduced energy expenditure [107, 108]. Before the molecular basis for obesity relating the *ob/ob* mouse model was established, speculation existed implicating an impaired peripheral signal that linked energy status to an adaptive response [109]. Positional cloning of the ob-gene finally led to the discovery of the proteohormone, leptin, which is predominantly produced by fat cells and the level of expression depends on the amount of fats stores. Leptin administration induces a negative energy balance mediated by neuronal structures in the hypothalamus and the brainstem [102].

Adaptive thermogenesis is suggested to play an important role in the development of obesity, as it accounts for much of the disparity in RMR that is observed between lean and obese individuals [110]. In rodents, interscapulary brown adipose tissue (iBAT) is the major site for adaptive thermogenesis, which in turn occurs in response to physiological or experimental stimuli such as cold exposure, birth, noradrenalin treatment, and consumption

of a hypercaloric diet [111]. In diet-induced thermogenesis, leptin activates (in the same manner as appetite regulation) POMC neurons in the arcuate nucleus of the hypothalamus through synapses with preganglionic neurons in the intermedial lateral column of the spinal cord, and with neurons in the PVN that are responsible for the control of sympathetic outflow from the central nervous system to the periphery [112, 113]. Activated POMC neurons release α-MSH which directly stimulates thermogenesis through the activation of the MC4-R located on sympathetic preganglionic neurons. Mice lacking the MC4-R have impaired diet-induced thermogenesis and are consequently obese [114, 115]. MC4-R activation stimulates sympathetic nerves leading to an increase in the release of norepinephrine and this significantly promotes sympathetic nervous system activity to brown adipocytes (see Figure 10) [106]. The release of norepinephrine from sympathetic nerve terminals subsequently leads to the activation of the β-3 adrenergic receptor on brown adipocytes [116]. These G-protein coupled receptors initiate an intra-cellular signalling cascade that ultimately results in the liberation of energy in the form of heat. The first stage in this pathway involves the activation of adenylyl cyclase which increases intracellular cyclic adenosine monophosphate (cAMP) levels, and in turn induces an increase in lipolysis and free fatty acids via protein kinase A. The availability of fatty acids triggers the uncoupling of cellular metabolism by uncoupling protein-1 (UCP-1) in the mitochondria to generate energy, which is released as heat [117-120]. UCP-1 gene expression has also been shown to be directly linked to the β-3 adrenergic receptor gene expression levels, suggesting that β-3 adrenergic receptor is responsible for the upregulation of UCP-1 gene expression.

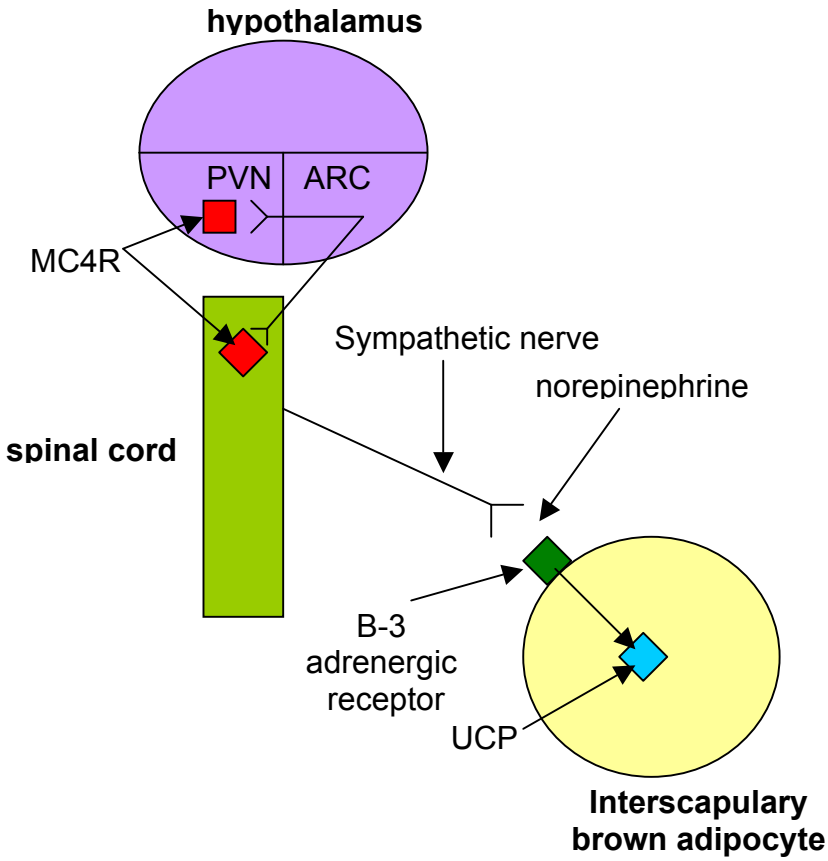


Figure 10. Schematic representation of sympathetic nervous stimulation of brown adipose tissue (iBAT). PVN: Paraventricular nucleus, ARC: Arcuate nucleus. UCP1: Uncoupling protein 1, MC4R: Melanocortin 4-receptor.

1.8.2. Cellular uncoupling

Reactions within the cell that generate energy are normally coupled [120]. This means that a given molecule of fuel generates a proportionate quantity of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH), equal to the number of protons that are transported out of the mitochondrial matrix by the electron transport chain. The protons re-enter the mitochondrial matrix via the mitochondrial membrane enzyme ATP synthase, creating a fixed amount of ATP that is proportionate to earlier events. This form of cellular metabolism is required to perform the basic housekeeping activities within each cell.

Cellular uncoupling (see Figure 11) is the process by which one of the stages of cellular metabolism is disrupted, preventing the operation of the normal metabolic cycle. During uncoupling, the energy that has been stored by the electrochemical gradient caused by the transport of protons out of the mitochondrial matrix is released as heat by UCP-1. UCP-1 thus uncouples the link between protons and ATP production, liberating heat while preventing the utilization of protons by ATP-synthase [121]. UCP-1 expression is exclusive to iBAT in rodents, and is primarily involved in heat production in response to cold exposure [121]. UCP-1 knockout mice have an inability to adequately regulate body temperature in response to cold exposure [122].

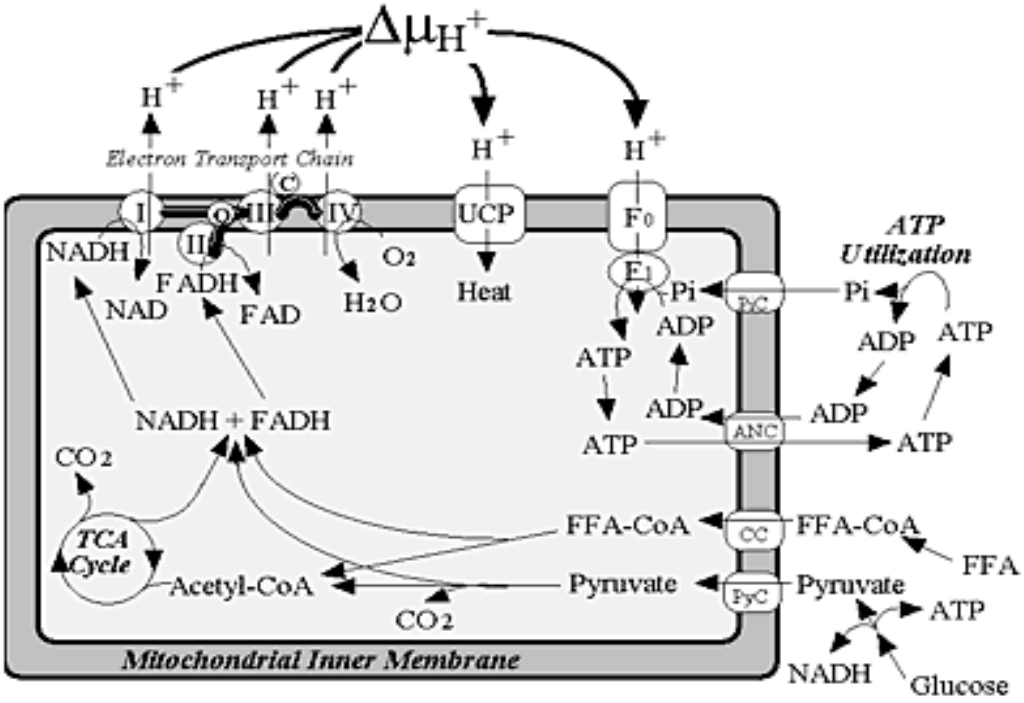


Figure 11. Schematic diagram of the cellular uncoupling process. Energy stored in the electron transport chain is dissipated as heat via uncoupling proteins, which uncouple energy stored in the electron transport chain from ATP production, yielding heat. NAD: Nicotinamide adenosine dinucleotide, FAD: Flavin adenine dinucleotide, ATP: Adenosine triphosphate, ADP: Adenosine diphosphate, FFA: Free fatty acids, FFA-CoA: Free fatty acid CoA, TCA cycle: Tricarboxylic acid cycle, PyC: Pyruvate carrier, CC: Carnitine carrier, Pi: Inorganic phosphate [106].

In addition to increasing body temperature following cold exposure, adaptive thermogenesis is also responsible for the increased body temperature following a meal.

This is termed diet-induced thermogenesis. A decreased energy efficiency, which is a more appropriate description of the process of cellular uncoupling, is known to be a significant barrier to the development of obesity [123].

1.8.3. Programming of energy expenditure

While the mechanisms associated with increased fat accretion following *in utero* growth restriction that characterize catch-up growth remain to be elucidated, there is increasing evidence implicating adaptive thermogenesis in this process. Studies have shown that catch-up growth is associated with increased susceptibility to central obesity and cardiovascular disease in later life [124, 125].

In a study in rats, the propensity for fat accumulation was observed in offspring from dams that were severely undernourished during the first 18 days of pregnancy [126]. Subsequent to this, another study was done wherein the offspring from undernourished dams were weaned onto a high-fat diet [26] and this has resulted in the amplification of the catch-up growth in these offspring. Although hyperphagia was observed it could only account for a proportion of the increased fat deposition observed in these offspring, suggesting a role for reduced energy expenditure [26]. Catch-up fat accumulation was found to be positively related to energy efficiency. .Energy efficiency refers to the body's ability to use energy and greater energy efficiency means that fewer calories are needed to perform basic tasks and more to energy storage in the form of fat. Energy efficiency refers to the body's ability to use energy. Thus, greater energy efficiency means that fewer calories are needed to perform basic tasks, and more can therefore be directed towards energy storage in the form of fat deposition. Any factor that reduces energy expenditure naturally increases energy efficiency. Defective thermogenesis may therefore explain the reduction in energy expenditure observed in rat offspring that have undergone catch-up growth following fetal nutrient restriction.

In another study, dams fed a protein restricted diet during gestation resulted in the birth of smaller offspring with limited fat reserves [127]. These offspring exhibited catch-up growth, in which accelerated growth occurred during the lactation and early post-weaning period. The extent of catch-up growth, however, was shown to be greatest in offspring weaned onto a balanced diet, as opposed to a restricted one. Fat accumulation increased considerably during this period suggesting increased energy efficiency, a notion supported by the food intake data showing no differences between offspring from protein-restricted and normally fed dams [127]. These observations clearly demonstrate a propensity for fat deposition in the offspring following a period of growth restriction *in utero*. Given that lipid synthesis uses a much larger amount of energy than protein synthesis [128], the depletion of fat in offspring at birth following intra-uterine growth restriction is expected. It has been noted in a previous study that fat reserves and endocrine responsiveness show greater sensitivity to the nutritional status during gestation and early post-weaning [129], following fetal malnutrition. This alludes to a mechanism inherent to fat tissue that promotes its replenishment following fetal malnutrition.

Several more recent studies that were undertaken in rats have provided evidence for a role of diminished adaptive thermogenesis following catch-up growth [130,131]. Rat offspring that were born small following *in utero* growth restriction, by subjecting pregnant dams to a protein-restricted diet, were fed post weaning a high fat or normal chow diet (i.e. control group). These offspring, that were growth-restricted *in utero* and weaned onto a chow diet, exhibited a similar rate of protein deposition (i.e. muscle growth) compared to control offspring. The accumulation of fat however was two times greater in the growth-restricted offspring compared with control offspring. Their energy expenditure was also reduced but their energy intake was no different from control offspring. This suggests that reduced thermogenesis might account for the altered energy expenditure responsible for increased

energy efficiency in the growth-restricted offspring. Feeding these offspring a high fat diet post weaning resulted in catch-up growth. Hyperinsulinaemia and hyperphagia were also observed in these offspring that were growth-restricted *in utero* compared to those fed post weaning the chow diet. Suppressed thermogenesis was suggested to be the basis for increased energy efficiency that favours not simply catch-up growth but more specifically to greater fat deposition, which I termed as 'catch-up fat'. It was also observed in these studies that fat deposition was the primary indicator of glucose tolerance, suggesting that the degree of hyperinsulinemia and insulin resistance observed in the growth-restricted offspring is dependent on reduced thermogenesis. It was also suggested that increased circulating cortisol levels contribute to the suppression of thermogenesis [130].

Catch-up fat is an appropriate response allowing for the replenishment of depleted fat reserves. While it allows early-growth restricted offspring to return to the normal growth trajectory, it has adverse ramifications [132, 133, 134]. An explanation for the suppression of thermogenesis from an evolutionary standpoint has been suggested, using the example of decreased resting metabolic rate (RMR) during fasting [135]. In such instances, humans and rodents increase energy efficiency in an attempt to efficiently utilize their limited energy supply. Metabolic requirements for undertaking given tasks are reduced and a sense of thrift prevails, whereby energy fuel storage is favoured over energy utilization. This metabolic setting mechanism is suggested to be utilised by intra-uterine growth-restricted (IUGR) offspring, whereby suppression of thermogenesis precedes fat accumulation and excessive weight gain particularly when dietary abundance replaces food shortages.

Studies have also suggested that a component related to fat stores modulates thermogenesis in response to body composition [129,131], and that this signalling mechanism persists during the period of catch-up growth to promote body weight gain and fat accumulation in response to their *in utero* environment. It is suggested that substrate cycling from lipid

oxidation to lipogenesis plays a role in accelerating fat accumulation through adaptive suppression of thermogenesis, with leptin and insulin implicated in this process [131]. Two independent regulatory systems for thermogenesis have been postulated. One is diet-induced thermogenesis, which is regulated by the sympathetic nervous system, and the other is a fat-specific modulator of thermogenesis, which is under the direct influence of fat reserves and responsible for suppression of thermogenesis during catch-up growth [131]. In addition to the suggestion of increased endocrine sensitivity following fetal malnutrition [129], it was observed that *de novo* cycling of glucose and fatty acid synthase hyperactivity promoted catch-up fat in growth-restricted offspring fed post weaning a high-fat [135]. Moreover, while skeletal muscle insulin-stimulated glucose uptake was impaired in these offspring, suggesting insulin resistance, glucose uptake into adipose tissue was increased. This would support the concept of increased endocrine responsiveness of adipose tissue.

A recent study has directly linked suppression of diet-induced thermogenesis with the development of obesity in a mouse model where offspring were exposed prenatally to maternal undernutrition and fed a post weaning diet high in fat [136]. The study in question suggested an alteration in the regulation of diet-induced thermogenesis in these offspring, possibly due to impaired sympathetic nervous system activity. These offspring went on to develop obesity in adulthood. The observation of a premature leptin surge in offspring that were malnourished *in utero* has been attributed to the later development of obesity [137]. It appears that in malnourished fetuses, the normal leptin surge is not observed. Hence, malprogramming of leptin-mediated thermogenesis occurs in these offspring, as shown by their impaired response to acute peripheral leptin administration. A study has confirmed the importance of an inappropriately timed leptin surge in the physiology of reduced energy expenditure, which promotes catch-up growth [138].

1.8.4. Programming of obesity by glucose and glucocorticoids

The deleterious health consequences of being large at birth have largely been established [139,140]. Thus elaborating on mechanisms contributing to increased birthweight may provide a basis for establishing the causes of later obesity. It is important to understand mechanisms other than those involved in energy expenditure which may be programmed and serve to promote obesity.

One possible mechanism for the fetal programming of obesity by overnutrition is through maternal diabetes. This is exemplified in the Pima Indians of Arizona, which has the highest incidence of type II diabetes of any population in the world and in whom the strongest risk factor for this condition is exposure to diabetes *in utero* [141]. Likewise, a study in rats has shown that offspring whose mothers were injected ip with glucose during pregnancy have increased susceptibility to type II diabetes [142]. These offspring are heavier and have larger fat depots due to the effect of insulin in promoting fuel storage, thus increasing their likelihood of becoming obese in later life. The case of gestational diabetes encompasses both genes and environment, as it is plausible that diabetic mothers are also likely to pass on the genes that made themselves susceptible to diabetes. Thus it is important not to consider gene and environment as mutually exclusive, but possibly involved in a synergistic partnership to the detriment of the offspring's health.

Glucose is the primary precursor for lipid synthesis [143], which explains why an increase in fetal plasma glucose (accompanied elevation of fetal insulin levels) leads to elevated lipid accumulation. Evidence suggests that promotion of adiposity through fetal exposure to elevated glucose levels might be influenced by previous exposure to nutrient restriction. Bispham et al. [130] demonstrated that the degree of adiposity due to fetal exposure to glucose excess is dependent on nutrient status of the mothers from early to mid-gestation. Thus, early gestation exposure to nutrient restriction with subsequent late gestation

manipulation of glucose levels by rapidly increasing fetal glucose supply, results in greater lipid deposition and increased fat depots at birth [130]. The authors of the study in question have postulated that insulin-like growth factors (IGFs) are involved in fetal adipose tissue accumulation [130]. IGF receptors, of which there are two main types namely IGF-1 and IGF-2, have been implicated in the development of fetal adiposity. It has been shown that there are increased levels of IGF receptors as an adaptation to early gestational undernutrition, an adaptation that would be inappropriate if glucose levels were raised in late gestation. The result would be an increased sensitivity of adipose tissue to the anabolic actions of IGF, at the time of glucose abundance, leading to a propensity for lipid synthesis. IGF-1 receptors are believed to be more metabolically influential than IGF-2 receptors, due to their prevalence in metabolically active tissue and organs. Studies have confirmed that deficiencies in IGF activity, potentially due to impaired IGF-1 receptor function, contribute to the small and thin phenotype of offspring subjected to intra-uterine malnutrition, and that a reversal of fetal levels of nutrition would have the opposite effect on IGF levels [144]. Thus IGF and its receptors may contribute to the high birthweight observed following fetal overnutrition or gestational diabetes.

1.9. Aims and objectives of this project

The precise peripheral mechanisms for reduced metabolic activity as well as altered central effects of appetite following *in utero* undernutrition and subsequent overnutrition remain to be elucidated. Although there is an increasing number of studies showing that food intake and metabolism are altered by undernutrition *in utero* followed by post weaning overnutrition, the precise mechanisms involved remain elusive. The general aim of this project is to identify some of the central and peripheral mechanisms that regulate metabolism and feeding, as a result of the aforementioned nutritional insult.

The fetal origins hypothesis proposes that adulthood hypertension, insulin-resistance and dyslipidemia, which are markers of the metabolic syndrome, originate in early development [12]. Low birth weight has often been used as a proxy for insufficient maternal nutrition during pregnancy. However, a recent study demonstrates fetal programming of altered cardiovascular function in adulthood, despite the absence of any changes in birthweight [145].

A variety of dietary manipulations during early development have been shown to induce fetal programming of adulthood disease phenotypes consistent with the metabolic syndrome. For example, maternal protein-restriction *in utero*, in which the protein content of the mother's diet during pregnancy is reduced, has been shown to promote insulin resistance, altered adipocyte development and cardiovascular dysfunction in rodent offspring [146-148]. On the other hand, maternal global undernutrition during pregnancy has been shown to result in impaired glucose tolerance and endothelial dysfunction [149,150]. Other mechanisms of *in utero* undernutrition such as placental insufficiency have been shown to negatively alter cardiovascular function in adult humans [151].

This thesis is primarily concerned in addressing three areas of fetal programming of adulthood metabolic and cardiovascular dysfunction: (1) to determine the importance of the type of undernutrition imposed on the dams, (2) to examine the effect of window-of-exposure to such nutritional manipulation to the dams and (3) to find out the effect of a dietary mismatch during prenatal and post-weaning period by exposing offspring post weaning to a high-fat diet. More specifically, this thesis aims to address the following questions:

1) What are the phenotypic differences between offspring from dams that were protein-restricted or globally undernourished during pregnancy?

2) To what extent does extending maternal undernutrition to include the lactation period affect the resulting phenotype of the offspring and their response to post-weaning high fat feeding?

3) How does post-weaning high fat feeding alter the phenotype of the offspring born to dams fed the protein-restricted diet during pregnancy alone or during pregnancy and lactation?

4) Are these phenotypic changes sex-specific?

Chapter 2

General Methodology

2.1. Animal model

MF-1 outbred strain of mice was used in these studies. They were purchased from a commercial source (Harlan UK). The use of the mouse as animal models to study developmental origins of disease is advantageous for a number of reasons. Their short gestation period and lifespan allows for an easily obtainable assessment of effects of gestational events on the adult offspring. The mouse genome has been studied extensively over recent decades, such that the majority of genes have now been mapped to their respective loci, providing invaluable information that is essential for gene expression analysis. All animal procedures undertaken were in accordance with the UK Animals Scientific Procedures Act of 1986.

2.2. Animal Husbandry

All animals were housed under a 12 hour light/dark cycle. The ambient room temperature was 22±2°C. They were provided with water *ad libitum* and unless otherwise stated in the feeding regimes were fed a standard chow diet *ad libitum*.

The number of experimental animals was established following an assessment of the potential for obtaining statistically significant interactions using the SPSS 9.0 statistical program. A sample size of 5 mice per group was estimated to be sufficient. The Following equation can be used to obtain the appropriate n size to ensure the desired power of a given study between two given groups.

$$n = \frac{(Z_{\alpha/2} + Z_{\beta})^2 (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

For the above equation, α: The maximum probability of committing a type 1 error (or simply the significance level, i.e. 0.05); β: the probability of a type 2 error = probability of not rejecting the null hypothesis if the null hypothesis is false (we use 0.20); Zα and Zβ: z-Statistic used to allow outcome of interest to be treated as if it were normally distributed

(we use $Z\alpha/2$: 1.96; $Z\beta$: 1.64). $\mu 1$: Mean of group 1, $\mu 2$: Mean of group 2. $\sigma 1$: Standard deviation of group 1; $\sigma 2$: standard deviation of group 2.

Time-mating of female mice with breeding males commenced immediately after the acclimatization period. Pregnancy was confirmed by the presence of a vaginal plug and regarded as day 0.5 of pregnancy. Mated dams were individually housed from the time pregnancy was confirmed through to birth and the lactation period. Unless otherwise stated all offspring were grouped-housed according to sex.

2.3. Dietary manipulation

2.3.1. Global undernutrition

Global undernutrition during pregnancy was undertaken by reducing the diet to 50% of the daily intake of the *ad-libitum* standard chow fed mice. In Chapter 3, a 50% global food-restriction was imposed on female mice on confirmation of their pregnancy (i.e. presence of vaginal plug) following mating to breeding males. On day 18.5 of gestation, chow diet was provided to prevent dams from eating pups at birth.

2.3.2. Dietary protein restriction

Dietary protein restriction has been successfully used in rodents, for reproducing physiological abnormalities, which are consistent with a thrifty phenotype in adult offspring challenged during intra-uterine development [152,153]. In my studies, I've used a 50% reduction in total casein content of the diet. Tables 1 and 2, show the dietary composition of the protein-restricted (PR) diet.

Table 1. Nutritional composition of chow and protein-restricted diets

Weight per 100g	Standard chow control (C) diet (4.17 kcal/g)	Protein Restricted (PR) diet (4.17 kcal/g)
Protein	18	9
Fat	20	10
Carbohydrate	62	77.8

Table 2. Specific ingredients of low-protein diet compared with standard chow.

Ingredients (g/100g diet)	Standard chow (C) control diet	Protein Restricted (PR) diet
Casein	18	9
Corn Oil	10	10
Starch	42.5	48.5
Sucrose	21.3	24.3
D-L-methionine	0.5	0.5
Choline	0.2	0.2
Cellulose	5.0	5.0
Minerals and Vitamins	2.5	2.5
Total	100	100

2.3.3. High fat feeding

The high fat diet was formulated to closely resemble the increase in fat consumption seen in humans. It is based on data from the National Diet and Nutrition survey that highlights the fact that at present men and women consume 30% more saturated fat and 25% more protein in terms of calorie content of the diet than the recommended daily intake [154]. This diet was purchased from a commercial source (RM1; Special Diet Services UK). Tables 3 and 4 show the nutritional composition of the high-fat diet.

Table 3. Nutritional composition of the high-fat diet

Components	High-fat diet (4.54 kcal/g) weight per 100g
Protein	20
Fat	45
Carbohydrate	35

Table 4. Specific ingredients of the high-fat diet

Ingredients	% Inclusion	g/kcal
Rice starch	28.34	22.97
Casein	26.53	21.50
Lard	17.89	14.50
Sucrose	10.49	8.5
Cellulose	6.17	5.0
Soya milk	4.32	3.50
Mineral mix	4.32	3.50
Vitamin mix	1.23	1.00
L-Cystine	0.40	0.32
Choline bitartrate	0.30	0.24

2.4. Monitoring food intake and weight gain

Food intake was measured by weighing the remaining food on the feeding through each day and topping them up thereafter. Individual body weight was measured on a weekly basis and an average weight of each experimental group was calculated each week.

2.5. Blood pressure measurement

Systolic blood pressure was measured with a pulse amplifier (IITC, Life Science, Woodland Hill CA) and a computerized BP monitor. The system measures systolic blood pressure photo-electrically by recording the cuff pressure at which the interrupted flow

returns to the tail. Animals are brought to the testing room, which was heated to 30°C to increase blood flow to the tail, and allowed to acclimatize for at least 20 minutes prior to testing. Animals were placed in a cylindrical restrainer and were covered with a drape to reduce the amount of stress artifact. Their tails were threaded through a ¼ inch tail cuff. They were restrained for five minutes before any experiments were performed to allow the animal to calm down. Tail pulse was detected through the sensor attached to the amplifier. BP measurements were started by inflation of the cuff to 200 mm Hg and then the pressure released by 3mm/second. The upper trace monitors cuff pressure and the lower trace monitors the pulse with fluctuations around the centre line appearing at the onset of the pulsations. The first onset of pulsations is taken as the systolic blood pressure. Five readings were taken for each animal and the mean value was calculated. Blood pressure recording was done at 12 weeks age in the male and female offspring. To minimize inaccuracy in the recordings, animals were allowed to become accustomed to being restrained for several days prior to actual blood pressure recordings.

2.6. Indirect calorimetry

Metabolism and respiratory quotient was measured by indirect calorimetry using the Oxylet Pulmonary Metabolic Monitoring System (Panlab SL Spain, see Figures 12 & 13). The system monitors oxygen (O_2) consumption and carbon dioxide (CO_2) production within a metabolic chamber in which animals are housed for the duration of the experiment, calculating respiratory quotient and providing an assessment of energy expenditure. All animals were placed in the metabolic chamber for calorimetry assessment between 3 and 5pm in the afternoon and were fasted 6 hours prior to the experiments to minimize the short-term effects of prior feeding on metabolism. Measurements were taken between 28-30 weeks.

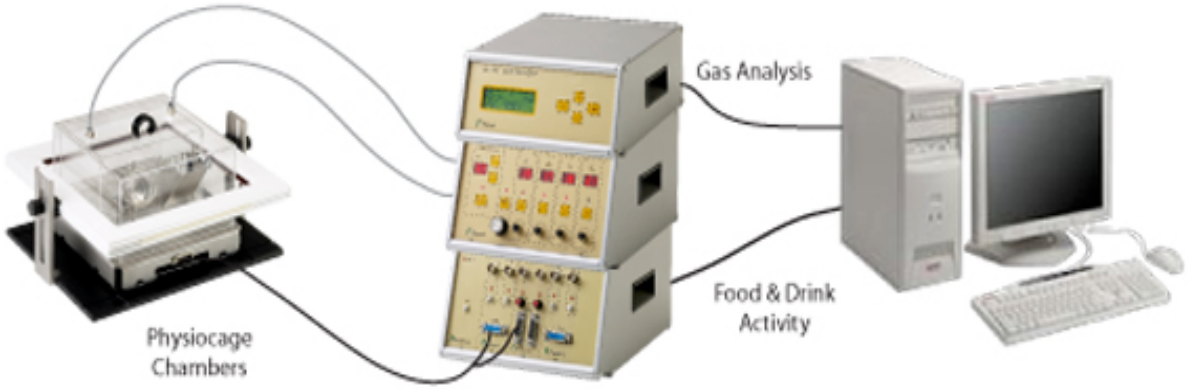


Figure 12. Pulmonary metabolic monitoring system (Oxylet) used for indirect calorimetry.

Figure 13. A mouse being housed in the metabolic chamber during calorimetry analysis.

2.7. Sampling protocols

2.7.1. Hypothalamic tissue dissection

The hypothalamic tissue block, the landmarks being the optic chiasma, lateral sulci, mammillary bodies, and a depth of 1 mm (Figure 14), was dissected from the offspring brain and snap-frozen in liquid nitrogen and stored at -80°C for future processing.

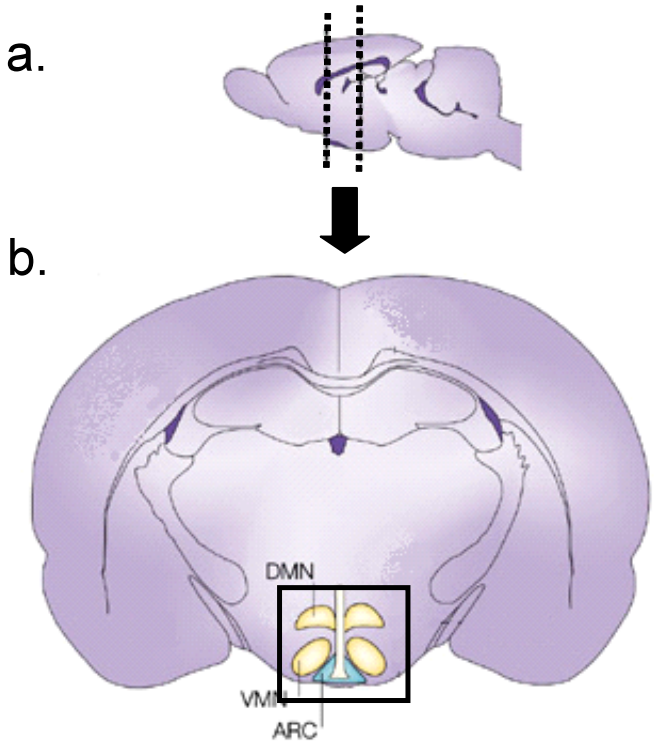


Figure 14. (a) Sagittal section through the mouse brain, showing the hypothalamic area of the brain taken during sampling (represented by the dotted lines). (b) Coronal section showing tissue block sampled (represented by the box). Based on Paxinos and Fraklin [155]

2.7.2. Dissection of fat pads

Individual fat pads in their entirety were dissected from the several fat depots (Figure 15)

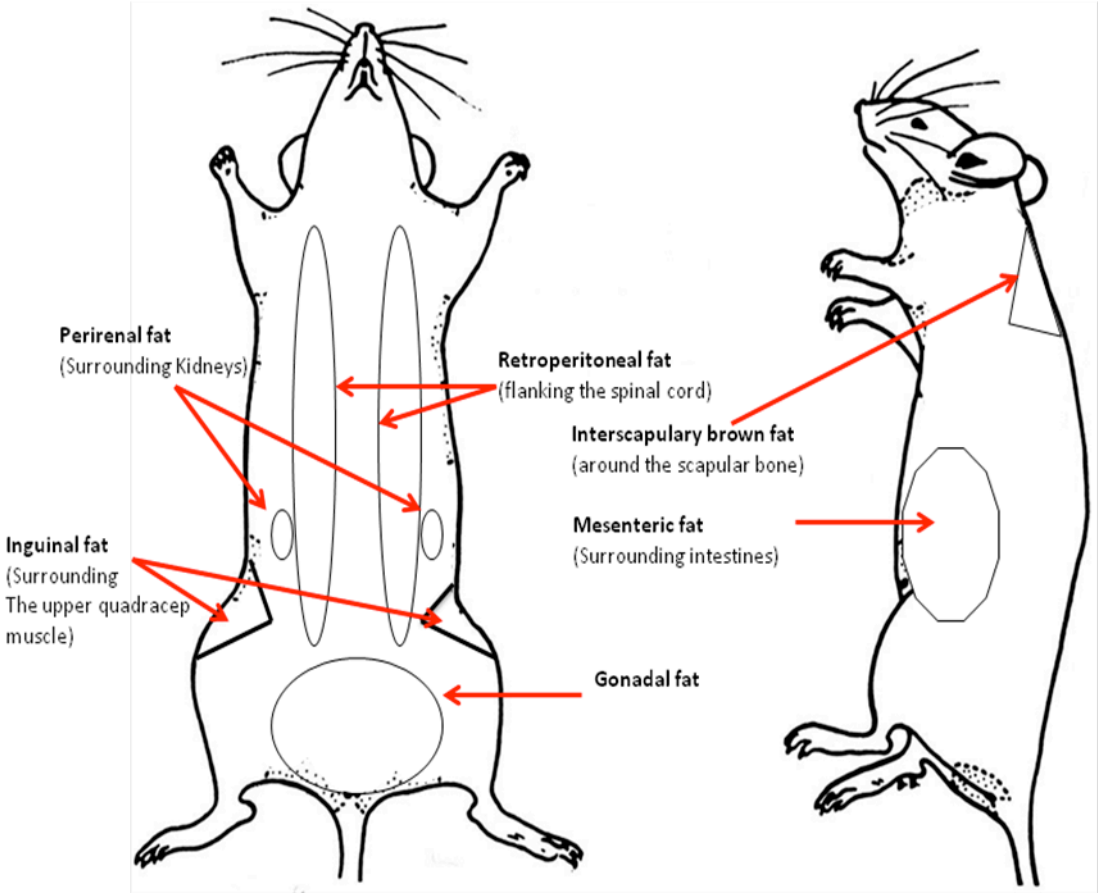


Figure 15. Diagram showing the region in which fat pads were dissected during sampling. Based on Smith and Schenk [156]

2.8. Processing of tissue samples for gene expression analysis

2.8.1. RNA extraction

Total RNA was extracted from tissue samples using the Sigma Tri-reagent™ method according to manufacturer's manual. Briefly, the tissue of interest was homogenised in Tri-reagent™, which is a guanidine thiocyanate and phenol solution that dissolves the DNA, RNA and protein following homogenisation. Chloroform was then added to separate the mixture into its RNA, DNA and organic phases. The RNA phase was removed and the RNA was then precipitated out by means of isopropanol and washed using 75% ethanol. The RNA pellet was then dried and then re-dissolved in ultra-pure water. The A260:A280

ratio for the samples was measured using a spectrophotometer and the total RNA concentration for each sample was calculated. Samples were then diluted to 0.5μg/μl. A260:A280 ratios of 1.40 and above were included in the experimental samples for cDNA synthesis and subsequent gene analysis.

2.8.2. cDNA synthesis

Once the RNA had been calculated, it was then used to synthesise cDNA, which was then used to examine expression levels of genes of interest that may have been involved in programming mechanisms. This was carried out using a variety of molecular biological techniques such as polymerase chain reaction (PCR).

For PCR, cDNA and not RNA is used due to its superior stability and relative resistance to degradation over time. Synthesizing cDNA from RNA also allows for increased amounts for starting material. For Real Time RT-PCR cDNA synthesis was carried out for the standard curve, positive and negative controls and samples to be analysed. An RNA sample (with the highest quality $A_{260/280}$ reading) was used as make up the standards. The RNA sample diluted to 0.5μg/μl was allocated as Standard 7(S7). A series dilution was then carried out, double diluting down to produce S6 then S5 and so on to S1. Two microlitres of each solution was taken for cDNA synthesis hence the amount of RNA used for the standard curve ranged from 1μg for S7 to 0.015625μg for S1. Five microlitres of the S7 RNA sample was removed and diluted a further 8-fold to produce a control solution that fell approximately within the middle of the standard curve. Two microlitres of this solution was removed six times for cDNA synthesis to produce six positive controls, C1 to C6.

The cDNA for negative controls, to monitor for possible contamination of RNA samples with genomic DNA, were produced using 1μg of RNA but in the absence of the MMLV

reverse transcriptase in the reverse transcription process. The samples to be analysed were diluted from 0.5μg/μl to 0.05μg/μl so they fell approximately within the middle of the standard curve. Two microlitres (i.e. 0.1μg) of this RNA was used for cDNA synthesis. For all the samples cDNA was synthesised using 2μl of the RNA in a volume of 25μl containing 0.4μg random oligo primers, RT-buffer, 12.5nM PCR mix (dNTPs), 25units RNAsin, 200units MMLV reverse transcriptase (Promega Southampton UK), and an appropriate amount of ultra-pure water. The RNA was first mixed with the random oligo primers and made up to 15μl with ultra-pure water, then heated at 70°C for 5 minutes then rapidly cooled on ice. The remaining reagents were then added and made up to 25μl with ultra-pure water. Samples were then incubated at 37°C for 1 hour, 42°C for 10 minutes and at 75°C for 10 minutes to stop the reaction.

2.8.3. Measuring changes in gene expression

The pattern of genes expressed in a cell is a feature of its current state and virtually all differences in cell state are correlated with changes of mRNA levels of many genes. Therefore as previously mentioned, the cDNA was then used to measure possible changes in gene expression in offspring exposed to a maternal PR diet using specifically-designed primers which bind to and allow for replication of a target area of the gene in question. There are a number of ways to do this including PCR, microarray, and *in-situ* hybridisation.

In contrast to microarray; real-time RT-PCR is low throughput with respect to the number of genes that can be analysed, usually one, but high throughput with respect to the number of samples that can be analysed, (i.e. 96 or more). Real-time RT-PCR has a high sensitivity and specificity, offers good reproducibility and only requires the use of small amounts of total RNA [157].

Microarray is a high throughput technology with respect to the number of genes analysed. Microarray allows hundreds to thousands of genes to be examined simultaneously, which in theory would facilitate the examination of the metabolic pathway. However microarray is low throughput with respect to the number of samples that can be analysed, often only one at a time. Furthermore there is a risk of generating false signals due to non-specific hybridisation and arrays may not be sensitive enough to detect rare gene transcripts.

In-situ hybridisation is a process that allows the identification of a specific nucleic acid sequence in its native location (i.e. *in-situ*). The first step of this process involves the gentle fixing of the desired tissue, giving it stability. Tissues are then hybridized to a chemically or radioactively labelled RNA or DNA probe that is complimentary sequence of interest. The chemical or radioactive probe can then be detected in the tissue by antibodies to the chemical label or by autoradiography. When using the chemically labelled probe, the antibody may be fluorescently labelled or can have an enzyme associated with it that when exposed to substrate generates a coloured precipitate, enabling localisation in cells. Real-time PCR has been regarded as a more reliable technique for gene quantification due to inhibitory effects of certain fixation and embedding techniques on accurate gene quantification [158].

2.8.4. Medium throughput quantitative competitive PCR

For medium throughput quantitative competitive PCR (MT qcPCR) cDNA was synthesised in a similar way. Half of a microgram of RNA was added to ultra-pure water for a final volume of 12.5µl. The RNA was then denatured at 70°C for 5 minutes. To this solution RT-Buffer, 0.5µg random oligo primers, 10nM PCR mix (dNTPs), 20units RNAsin and 200 units MMLV reverse transcriptase (Promega Southampton, UK) were added and made up to a final volume of 25µl with ultra-pure water. These samples were then incubated at 42°C for 1 hour, and then 95°C for 5 minutes to denature the MMLV enzyme and thus stop the reaction. MT qcPCR is also known as competitive PCR and it

involves the use of standards of known concentrations in order to determine the concentration of target DNA. Figure 16 shows a diagrammatic representation of the MT qcPCR. MT qcPCR confers the ability to measure several genes simultaneously. It also provides the additional benefit of being a more cost-effective way of measuring genes compared to Real-time PCR [159].

There are several sources of variation in RT-PCR that must be controlled. The RT reaction must be monitored to ensure efficient transcription. There is a risk of small differences in the early cycle periods during amplification producing dramatic differences in the yield of DNA that could hamper attempts to quantify results. These differences have been attributed to tube variations and/or variations in the temperature throughout the block of the cycler. Variation can also be introduced due to differences in RNA loading or quality of RNA [160].

To control for these variations different internal standards have been designed. Internal standards can be co-amplified with the target sequence in order to control the efficiency during amplification. Internal standards can be divided into two main categories, endogenous internal standards and exogenous internal standards [160].

Endogenous internal standards are normally sequences thought to be present at constant levels throughout the samples to be prepared. Housekeeping genes such as β-actin, GAPDH, 18s ribosomal RNA or cyclophilin are normally used as endogenous internal standards. Although they are useful in controlling for differences in RNA loading and differences in the quality of RNA they have limitations. The efficiency of the PCR for the control and target sequence must be as close as possible so one can be confident that the differences obtained are due to differences in the number of molecules at the start of the reaction and not due to differences in the PCR efficiency. However the different primer

sequences used for target and internal standard could potentially create different PCR efficiencies. Furthermore, housekeeping genes are commonly found at higher concentrations than the target message, thus products of the standard accumulate at earlier cycles than target products. This creates difficulties if the same number of cycles must be used for both products [160].

Exogenous internal standards can be either a synthetic RNA sequence or a synthetic DNA sequence that is not present in the target sample. In this study, a synthetic DNA sequence was used as the standard. The most frequently used standards are homologous or heterologous DNA sequences that contain the same primer sequence as the target and therefore compete with the target for the same primers. When a homologous internal standard is used, a restriction site or sequence is added or deleted so the standard is a different size from the target and can thus be differentiated from the target by electrophoresis [160].

As well as controlling for variation, internal standards also allow the number of transcripts present in the target sample to be quantified. This can be done by using several different concentrations of standard with the target sample in which the quantity of total RNA was kept constant. The number of molecules in the target sample could then be extrapolated by the number of molecules in the standard that gave a ratio of 1.0 to the target gene during amplification. For this the concentration of the standard must be known [160]. When using this method it is important to show a linear correlation of the target/standard ratio vs. different concentrations of standard. It is also important to show that the slope produced is a slope of 1.00, which assures that there are no dramatic differences in the efficiencies between the target and standard sequences [160].

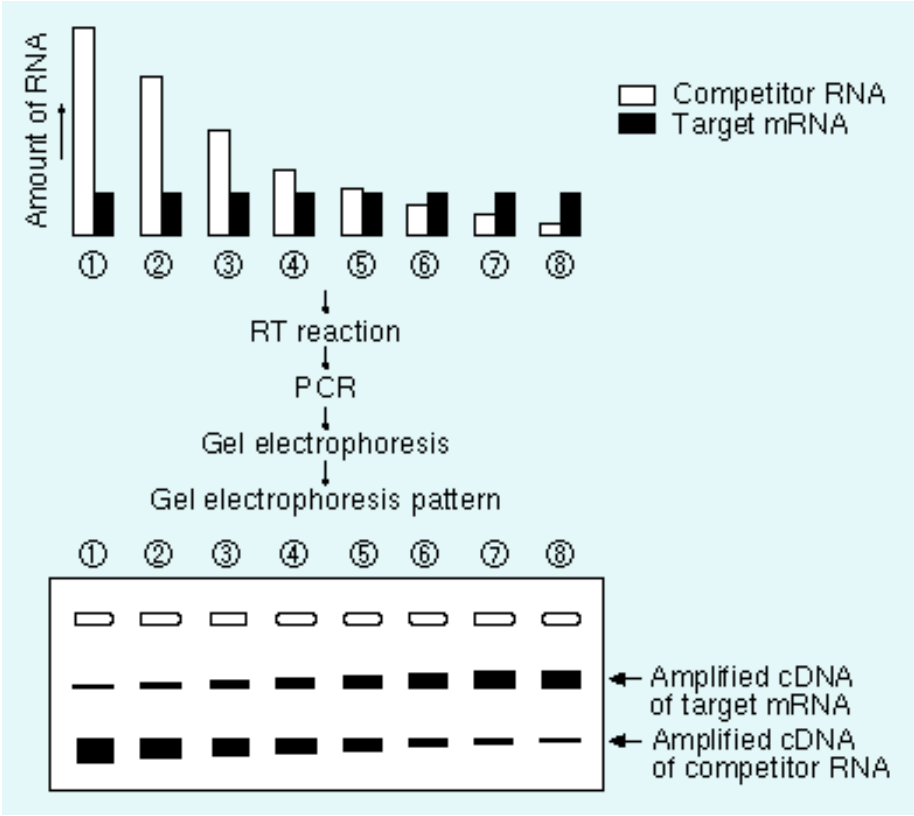


Figure 16. An example of how competitive PCR can be used to determine the target concentration. A known amount of various standard concentrations is compared with varying amounts of total RNA.

2.8.5. Real time PCR

PCR involves numerous cycles of DNA denaturation at a certain temperature followed by annealing and extension of the DNA at a lower temperature. This leads to an exponential increase in the number of copies of the gene of interest. At the beginning of a PCR reaction, amplification can proceed at a constant exponential rate as reagents are in excess and template and product concentrations are low enough to not cause renaturation and not compete with primer binding. Eventually product renaturation that competes with primer binding occurs and causes the reaction rate to go from an exponential phase of amplification to a linear phase and at a later time point the amplification rate plateaus. Thus, for accuracy, it is necessary to obtain data at a point in which every sample is in the exponential phase of amplification. This is known as the cycle threshold (Ct).

In real-time RT-PCR expression levels of a gene can be determined using a standard curve. The standard curve is constructed from RNA of known concentration and is then used as a reference standard for extrapolating expression levels of target mRNA. To minimize variability due to variable RNA inputs and integrity results can be corrected by normalisation to a housekeeping gene such as β-actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or to 18s ribosomal RNA. Figure 17 is a diagrammatic representation of the various stages of real-time PCR.

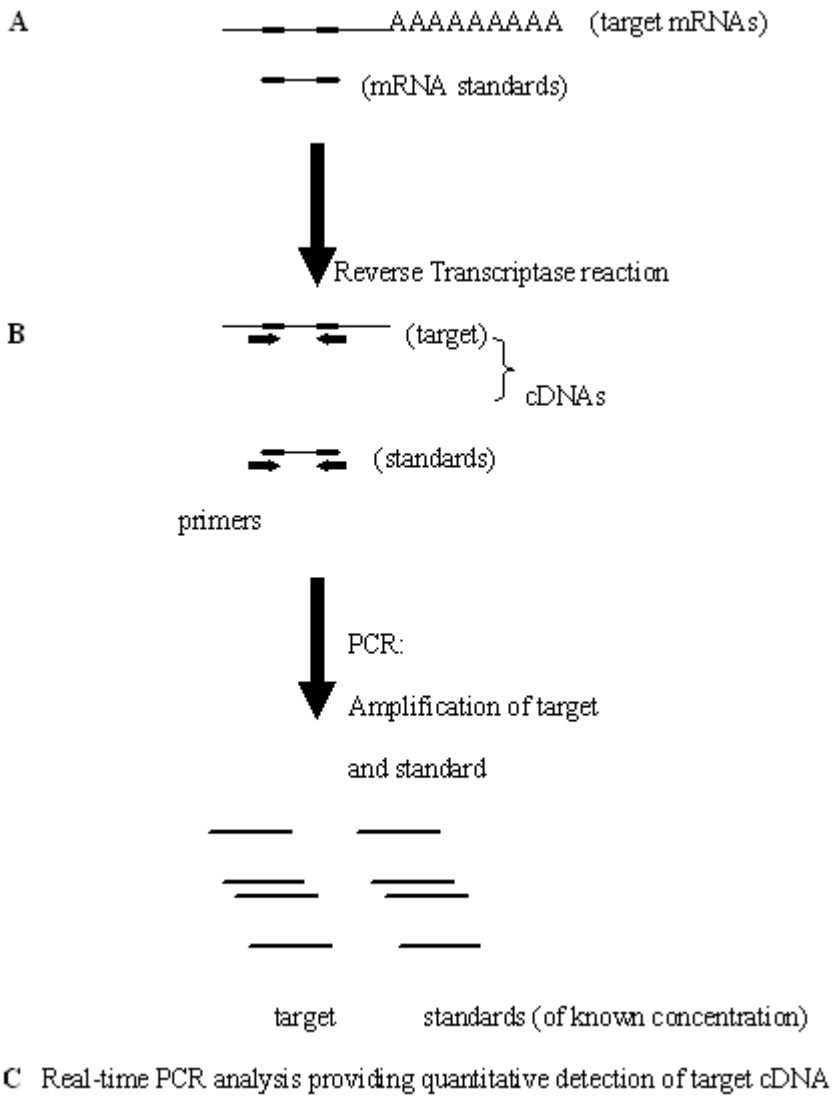


Figure 17. Flow-chart showing the stepwise molecular protocol for quantitative PCR analysis.

2.8.6 PCR detection system

PCR products can be detected by the generation of a fluorescent signal. The most popular fluorescent-generating reagents are SYBR® Green (Molecular Probes) and TaqMan® probes (Applied Biosystems). SYBR Green provides a simple, sensitive and inexpensive method for detecting PCR products. SYBR Green binds to double stranded DNA and fluoresces upon excitation. Therefore as the double stranded PCR product accumulates fluorescence increases. However, SYBR Green will bind to any double stranded DNA in the reaction, including primer-dimers and non-specific reaction products, which may affect the accuracy of the results. TaqMan probes are oligonucleotides with a fluorescent reporter dye attached to the 5' end and a quencher dye attached to the 3' end. These probes are designed to be complementary to sequences within the PCR product. During the reaction the probe hybridises with the target sequence between the specific forward and reverse primers. During replication the 5' nuclease activity of the DNA polymerase cleaves the probe separating the reporter dye from the quencher dye, which results in fluorescence. During the exponential phase the increasing fluorescence signal is directly proportional to the initial amount of target mRNA in the sample. The present thesis utilized both real-time PCR and MT qcPCR for quantification analysis.

2.8.7 Designing and optimizing probes and primers for PCR

Specific primers and probes for genes of interest were designed based on their published sequences using the Primer Express™ (v1.0) software. Oligonucleotide sequences were synthesised by Eurogentec Ltd (Romsey UK). Primers and probes were reconstituted in ultra-pure water and a 10μM aliquot was made from the stock solution. Primers and probes were then optimised to establish their best working concentration. For primer optimisation PCR was carried out using samples that each had a different concentration of forward and reverse primers. Primers at optimum concentrations display a low Ct (cycle threshold) value and a high delta Rn (fluorescent signal) value. For probe optimisation PCR was

carried out using samples with varying probe concentration. Again at optimum concentration the probe will produce a low Ct value and a high delta Rn value (see Fig 18 and 19).

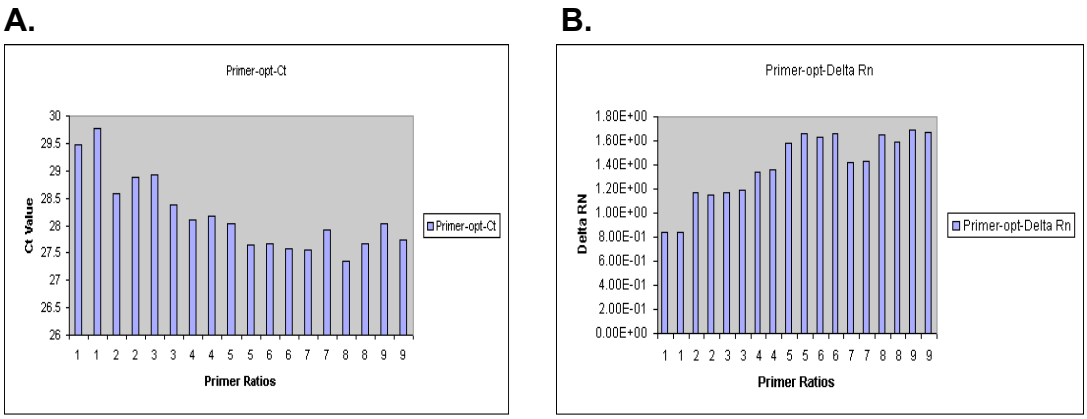


Figure 18. Example of primer optimisation. Primers at optimum working conditions will produce a low Ct value (A) and a high delta Rn value (B). In this example primer ratio 5 (300nM: 300nM) best fits these criteria.

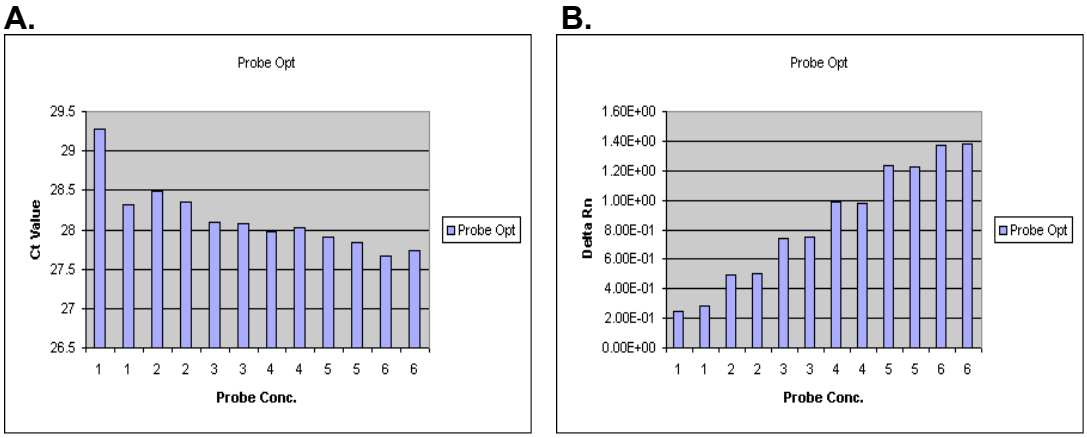


Figure 19. Example of probe optimisation. A probe at optimum working conditions will produce a low Ct value (A) and a high delta Rn value (B). In this example primer concentration 6 (150nM) best fits these criteria.

2.8.8. PCR protocol

For each sample 4µl cDNA was mixed with 50µl qPCR™ master mix (dNTPs, Hot Goldstar DNA polymerase, MgCl2, Uracil-N-glycosidase, stabilisers and passive reference), forward and reverse primers and probe at their optimum concentration and

made up to a final volume of 100µl with ultra-pure water. For the no-template control (NTC) cDNA was omitted and replaced with 4µl ultra-pure water. 45µl was dispensed in duplicate onto a PCR plate. PCR amplification was performed for 40 cycles. PCR consisted of initially 2 minutes at 50°C and 10 minutes at 95°C. This was followed by denaturation for 15 seconds at 95°C and annealing for 1 minute at 60°C, which were repeated for 40 cycles. An amplification plot (see Figure 20) of the increasing fluorescence signal for each sample was calculated using the ABI PRISM 7700 Sequence Detection System (SDS) software (v1.9).

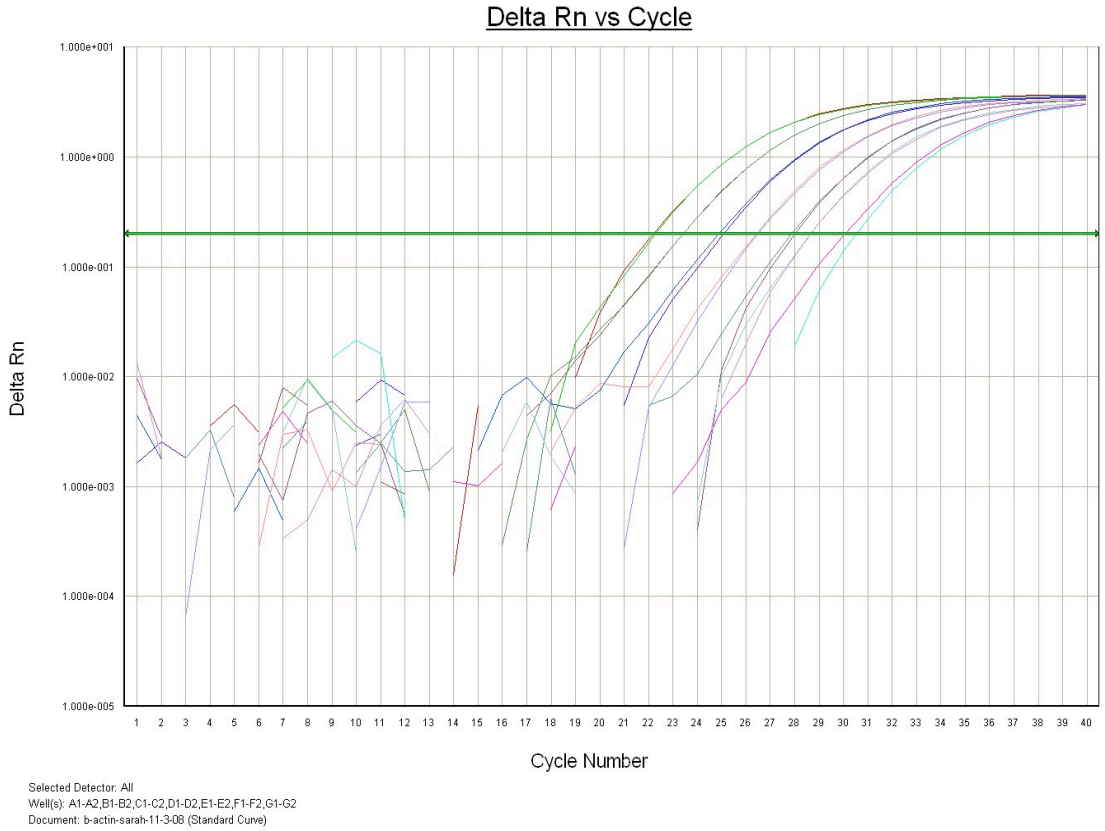


Figure 20. An example of an amplification plot produced by real-time RT-PCR for β-actin.

This is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter

Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Data was collected at a threshold point in which every sample was in the exponential phase of amplification.

A standard curve was constructed (see Figure 21). This is constructed from RNA of known concentration and is used as a reference standard for extracting expression levels of target mRNA. Standard-curves were reproducible, with CV (coefficient of variation) of less than 10%, and Pearson correlation coefficients above 0.980 for most comparisons. To reduce inconsistency owing to variable RNA inputs, values were corrected by normalisation to the housekeeping gene, β-actin or GAPDH.

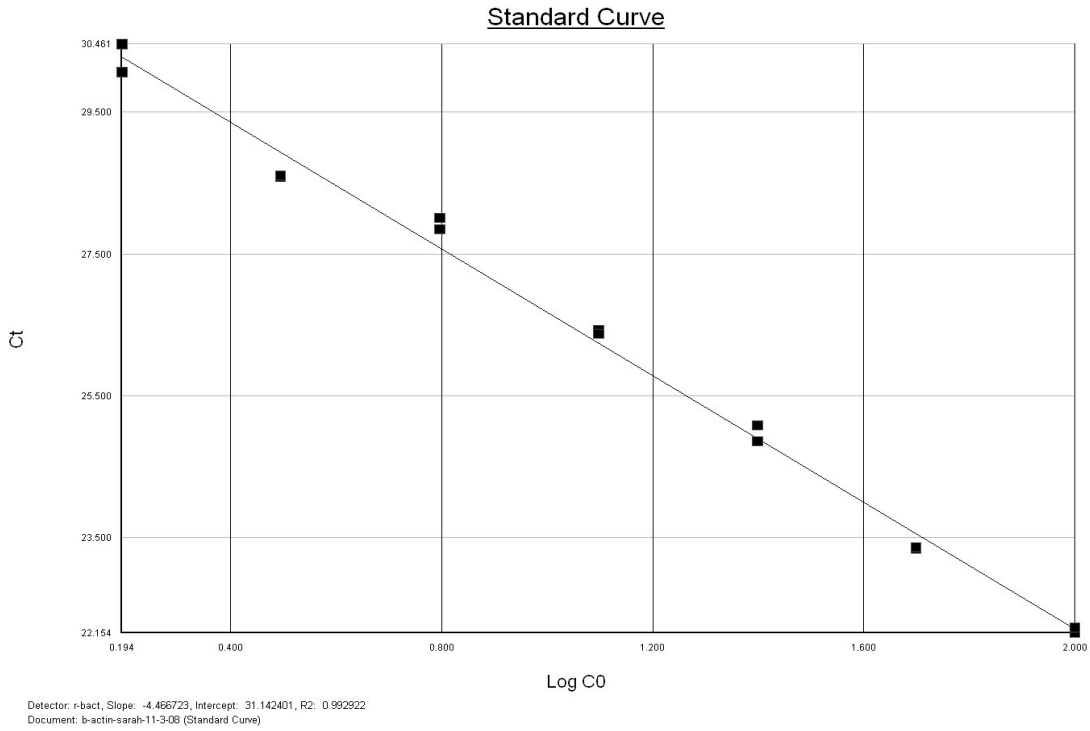


Figure 21. An example of a standard curve for β-actin which is constructed from RNA of known concentration and is then used as a reference standard for extrapolating expression levels of target mRNA.

The mRNA values for the samples of interest were then extrapolated from this, using the following equation:

mRNA value = 10^ ((Ct - c)/- m)

Where c and m come from y = mx+c equation from standard curve. Ct is the threshold cycle used to obtain data at which every sample is in the exponential phase of amplification.

2.9. Statistical analysis

All data is expressed as means ± SEM. Effect size estimates for body weight gain and energy intake are from a mixed model analysis [161] that considers all time points through the study, controlling for the set of dam-pup relationships. Differences in levels of gene expression between dietary groups at particular time points were evaluated using Students T-test or Mann-Whitney U test. Analysis of variance (ANOVA) is a general method for studying sampled-data relationships [162]. The method enables the difference between two or more sample means to be analysed, achieved by subdividing the total sum of squares. The purpose is to test for significant differences between class means, and this is done by analysing the variances. The basis of ANOVA is the partitioning of sums of squares into between-class and within-class. It enables all classes to be compared with each other simultaneously rather than individually; it assumes that the samples are normally distributed.

In order to establish exactly which group means are significantly different from which, a post-hoc test must be conducted following ANOVA in which statistical significance was found between a set of groups. The post-hoc test allows the limitation of error by precisely determining where statistical significance exists. Differences in levels of gene expression within dietary groups at different time points were evaluated using one-way ANOVA followed by Tukey-Kramer test for comparison where appropriate using the Sigma Stat (Systat Software Inc) or Prism (GraphPad Software Inc) statistical programs. A value of $p<0.05$ was considered significant. Repeated measures ANOVA, in which all members of

a sample are measured under different conditions. In some instances we also conducted a 2-way ANOVA to ascertain the relative importance of treatment one (e.g. PR diet) vs. treatment 2 (e.g. high fat diet) and to determine and prenatal-post weaning dietary interactions.

Chapter 3

The effects of maternal undernutrition during pregnancy and a post weaning high fat diet on the metabolic and cardiovascular phenotype of the offspring

3.1. Introduction

Maternal global undernutrition is a severe form of nutrient restriction during development compared to restricting specific macronutrients. In humans, global undernutrition occurs commonly in developing or Third World countries or during times of political turbulence and environmental disasters. In the Dutch winter famine of 1944, a combination of a blockade of all food transports to the Netherlands by the Germans and an unusually cold winter (the canals froze becoming impassable by barges) lead to food rations dropping to under 1000 calories per day [163]. This is less than 50% of the daily calorie allowance of a human adult. Pregnant women however, require up to 24,000 calories per day in the final trimester of pregnancy, which equates to over 10 times the recommended daily calorie allowance. This severe shortage of calories during pregnancy may explain the severely reduced birthweights of infants born during this famine [164]. The Great Chinese Famine that occurred between 1958 and 1961, which was a result of natural disasters and compounded by policy errors by the communist government, saw a similar reduction in daily calorie intake but over a much longer time period [165]. This famine lead not only to significant fatalities in the adult population but also significantly increased rates of miscarriage and still-births [166], reflecting the severity of caloric restriction.

Maternal caloric restriction by global undernutrition during gestation has been shown to induce an obese phenotype and alter the levels of certain genetic markers of metabolic and cardiovascular disease in rats [150]. However, the causes of obesity were well less defined than those associated with cardiovascular dysfunction. In one study [167], reduced expression levels of 11β-hydroxysteroid dehydrogenase (11βHSD), an enzyme involved in glucocorticoid regulation were found in rat offspring from dams subjected to a 50% reduction in their food intake during the last trimester of pregnancy [167]. Another study also showed that offspring from food-restricted dams had increased fat accumulation and size of adipocytes in their fat depots [168]. These results suggest that offspring exposed to

maternal undernutrition during development have become more energy efficient and have adapted to a perceived postnatal environment of undernutrition by storing energy in the form of fat. As discussed in Chapter 1, a reduction in 11βHSD expression may indicate an impairment of the HPA axis and IGF pathways associated in regulating cortisol and insulin secretion. The circulating cortisol and insulin milieu not only impact on muscle metabolism but also on fat deposition and accretion [76]

While obesity in adulthood may be attributed to undernutrition *in utero*, a dietary mismatch in which prenatal undernutrition is proceeded by postnatal overnutrition has been shown to further increase the risk of developing obesity [97,169]. The thrifty phenotype hypothesis states that fetal malnutrition acts as a biological cue resulting in alteration in the physiology of the developing fetus that allow for survival in a nutrient-depleted environment [170]. This hypothesis was further expanded to suggest that the fetus makes developmental responses *in utero* to ensure survival and reproductive fitness in its predicted postnatal environment [20]. If the actual environment differs from what is predicted, impaired physiological homeostasis and increased risk of disease can result. The predictive adaptive response phenomenon was demonstrated in rats where offspring from dams fed a protein restricted diet during pregnancy exhibited enhanced insulin sensitivity [171]. However when given a high fat diet in adulthood, these animals showed greater insulin-resistance compared with offspring from chow-fed control dams similarly weaned onto the high fat diet. In a similar study conducted in pigs, *in utero* exposure to a pro-atherogenic diet seems to have a protective effect on the offspring from developing cardiovascular disease when fed postnatally with the same pro-atherogenic diet [172]. Offspring exposed *in utero* to the pro-atherogenic diet but weaned onto a control diet exhibited aortic fatty streaks and increased risk of hypertension.

A study in mice, in which dams were restricted to 70% of the food consumed by the corresponding control group during the final 8 days before birth followed by consumption of a high-fat diet (35% kcal fat) by offspring half-way through adulthood (9 weeks of age), produced greater weight gain and obese phenotype in their offspring [173]. These offspring also had reduced diet-induced thermogenesis, as measured by indirect calorimetry. There was however no measurement of the cardiovascular status in these offspring. Given that a 30% global undernutrition was only imposed in the latter part of pregnancy and that the offspring were challenged with a high fat diet at 9 weeks of age, we refined this nutritionally mismatched experimental paradigm and examined its effect on the metabolic and cardiovascular phenotype in the offspring. In this part of my study, I subjected the dams to 50% global food-restriction, mimicking a similar calorie deficit that has been widespread in countries such as India and Brazil in the past 50 years. I also challenged the offspring with a high fat (HF) diet from weaning to adulthood to produce a maternal-offspring dietary mismatch. The metabolic and cardiovascular phenotype dietary mismatch was compared in the proceeding chapters with a mismatch which involves the modification of specific macronutrients in the maternal diet (i.e. protein content), while maintaining the same high fat dietary challenge to the offspring.

3.2. Aims

1) To determine the effects of a maternal caloric restriction by global undernutrition during pregnancy on the metabolic and cardiovascular phenotype of the offspring

2) To determine the effect of amplifying the dietary mismatch by feeding the offspring post weaning a diet that is high in fat.

3) To determine whether there are sex-specific outcomes in terms of the metabolic and cardiovascular phenotype in these offspring.

3.3. Methods

All procedures were carried out in accordance with the UK Animals Scientific Procedures Act of 1986. Female MF-1 mice were individually housed under a 12h light-dark cycle and given full access to drinking water throughout the study. At ages 8-10 weeks of age these females were time-mated and on confirmation of pregnancy were randomly divided into two experimental groups (see Figure 22). Half of the pregnant dams (n=5) had free access to a standard chow diet throughout pregnancy and designated as the *ad libitum*-fed (AD) group. The other half of pregnant dams (n=5) had a 50% reduction in their normal daily food intake during pregnancy and was designated as the undernourished (UN) group. The reduction in food intake was based on the *ad libitum* daily food intake of the C pregnant dams. The UN dams were however given 2.5g extra pellet on day 18.5 of pregnancy to prevent them from eating their own pups following birth as described previously [173]. The average duration of pregnancy was between 19 to 21 days. Litter size was standardized to 8 pups per litter. From birth to weaning (three weeks) all dams were fed the standard chow (C) diet. From weaning to sampling at 32 weeks of age, offspring were either fed the C or a high fat (HF) diet (see General Methodology in Chapter 2 for composition of the HF diet).

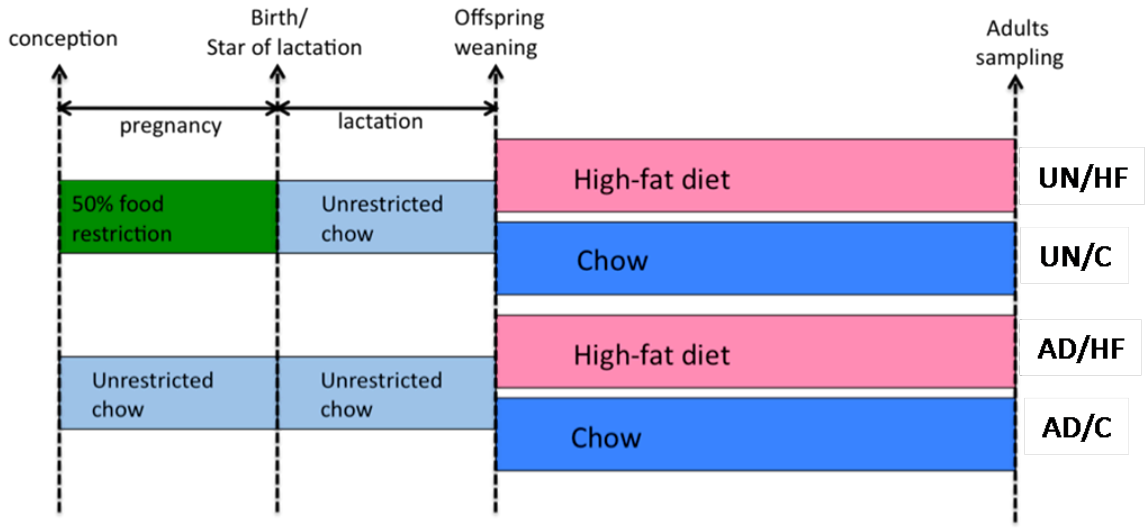


Figure 22. Experimental protocol for the global undernutrition study. This study generated 4 offspring treatment groups: those from dams that were undernourished during pregnancy (UN) and fed either a high fat (HF) or a standard chow (C) diet to adult (generating dam/offspring dietary groups UN/HF and UN/C), or from mothers fed *ad libitum* (AD) the C diet and fed post weaning C or HF diet to adulthood (AD/C and AD/HF).

Food intake and body weight of the offspring were monitored during the course of the study. Between 28-30 weeks of age the offspring's metabolism and blood pressure were measured by indirect calorimetry and tail-cuff plethysmography, respectively (see General Methodology in Chapter 2 for details). Adult offspring were humanely killed at 32 weeks of age by CO_2 inhalation and cervical dislocation. Each offspring group had a sample size of between 5-8 animals. Blood was collected by cardiac puncture and fat depots (i.e. gonadal, retroperitoneal, interscapular, inguinal and peri-renal) were dissected and weighed (see General Methodology in Chapter 2 for anatomical details). Cumulative fat depot weights for each animal were compared to total body weights and body fat as a percentage of total body weight was calculated

All values are presented as mean ± SEM. Effect size estimates for body weight gain and energy intake are from a mixed model analysis [161] that considers all time points through the study, controlling for the set of dam-pup relationships. All other data were analyzed

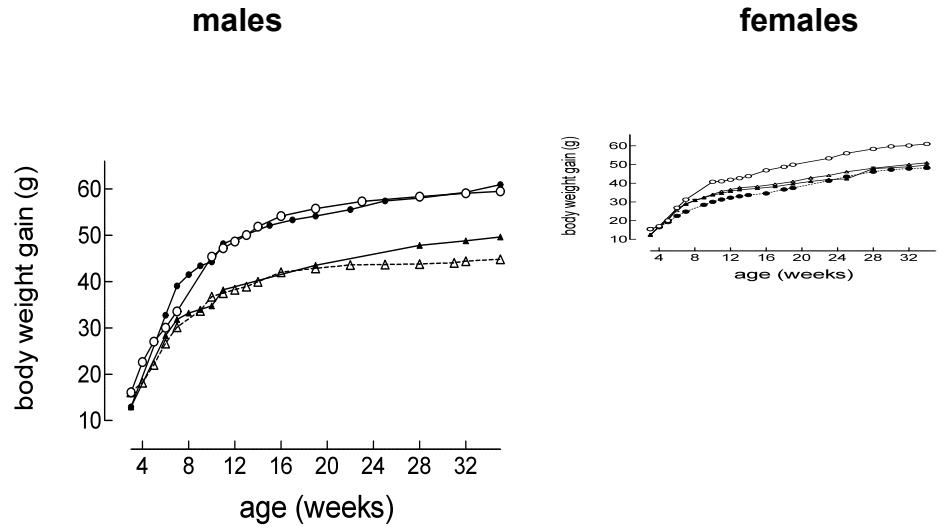
statistically using analysis variance (ANOVA) followed by the Tukey-Kramer test for comparisons where appropriate. Statistical significance was assumed if $P<0.05$. Mixed-model analysis was done with SPSS 14.0 (SPSS Inc). All other analysis was done using Sigma Stat (Systat Software Inc) or Prism (GraphPad Software Inc) statistical programs.

3.4. Results

3.4.1. Body weight and food intake

There was no difference in food intake between groups in either male or female offspring (see Figure 23 and Table 5). Regardless of maternal nutrition during pregnancy and post weaning diet, offspring consumed a similar amount of calories per day. In terms of bodyweight, both HF-fed male offspring were heavier ($p<0.001$) than their C-fed counterparts (see Table 5). There was no difference in weight between UN/HF and AD/HF offspring. However, UN/C male offspring were heavier than AD/C animals ($p<0.05$). In females, AD/HF offspring were considerably heavier then the remaining three offspring groups, namely UN/HF, UN/C and AD/C ($p<0.05$) (see Figure 23 and Table 5).

A.



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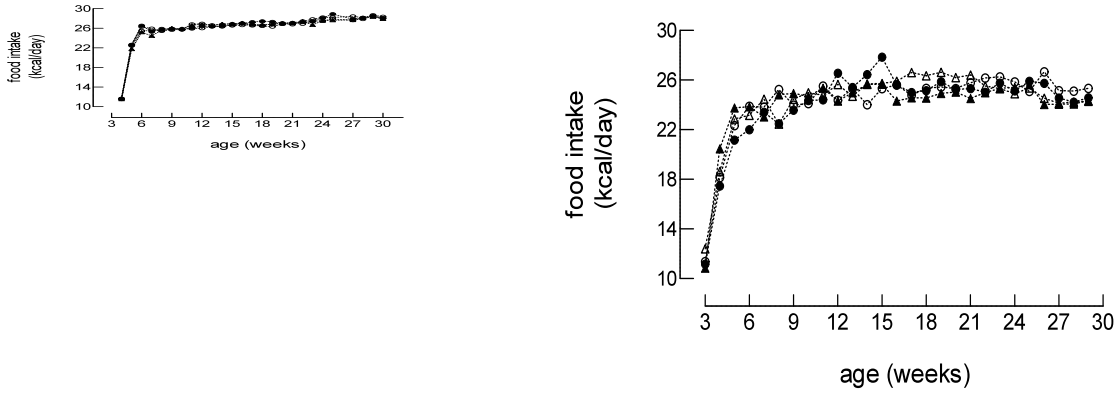


Maternal diet/offspring diet: △ AD/C ○ AD/HF ▲ UN/C ● UN/HF

Figure 23. Bodyweight trajectory (A) and Food intake (B) and in male and female offspring, respectively, from dams that were undernourished (UN) or fed the chow diet *ad libitum* (AD) during pregnancy and weaned onto either a high-fat (HF) or chow diet (C) to adulthood (n=5-8 per treatment group).

Table 5. Estimate of mean difference and 95%CI of body weight and energy intake in the offspring

Group comparisons		Variables			
		body weight, g		energy intake, kcal/day	
		males	females	males	females
UN/HF vs AD/C	Mean	5.08	0.21	0.4	0.11
	95% CI	2.34, 6.95	0.09, 1.12	-0.18, 1.57	-0.15, 1.03
	P value	<0.001	<0.001	ns	ns
UN/HF vs AD/HF	Mean	0.46	4.57	0.07	0.04
	95% CI	-0.19, 1.25	0.4, 6.78	-0.23, 0.61	0.74, 0.83
	P value	ns	<0.001	ns	ns
UN/C vs AD/C	Mean	1.35	0.04	0.10	0.22
	95% CI	-0.01, 1.96	-0.74, 0.93	-0.61, 0.49	-1.0, 0.64
	P value	<0.001	ns	ns	ns

Effect estimates (*n* =5-8 per group) are from a mixed model analysis that considers all time points through the study, controlling for the set of dam-pup relationships. ns = no significant difference.

3.4.2. Energy Expenditure & Respiratory Quotient

Energy expenditure (kcal/day) was reduced by 10% and 15% in UN/HF male offspring compared to AD/HF males ($p<0.05$) and AD/C males, respectively ($p<0.05$). No significant differences were observed between groups in female offspring.

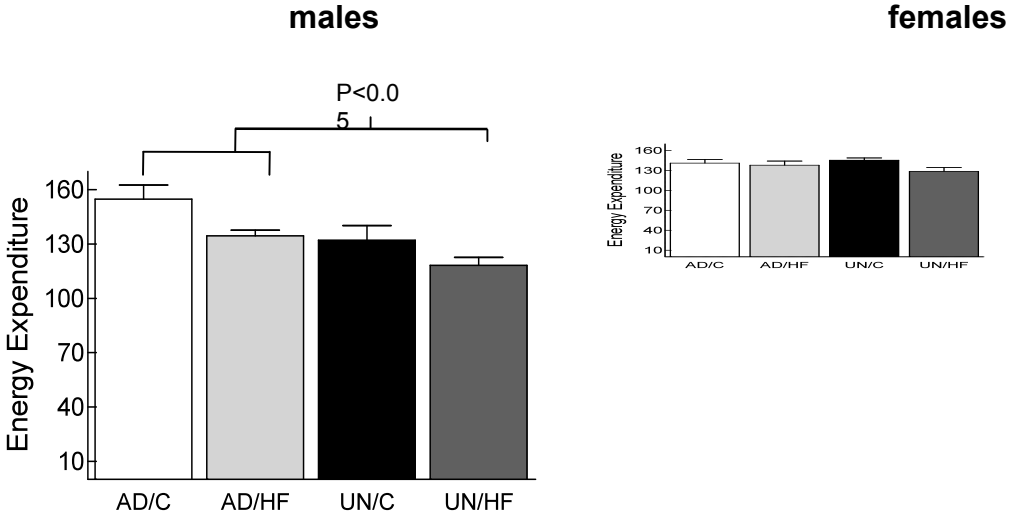


Figure 24. Energy expenditure in male and female high-fat (HF) or chow (C) fed offspring from dams that were either undernourished (UN) or fed ad-libitum (AD) during pregnancy (n=5-8 per treatment group).

Respiratory quotient was lowest in HF-fed offspring regardless of maternal diet during pregnancy and the sex of the offspring ($p<0.001$, Figure 25).

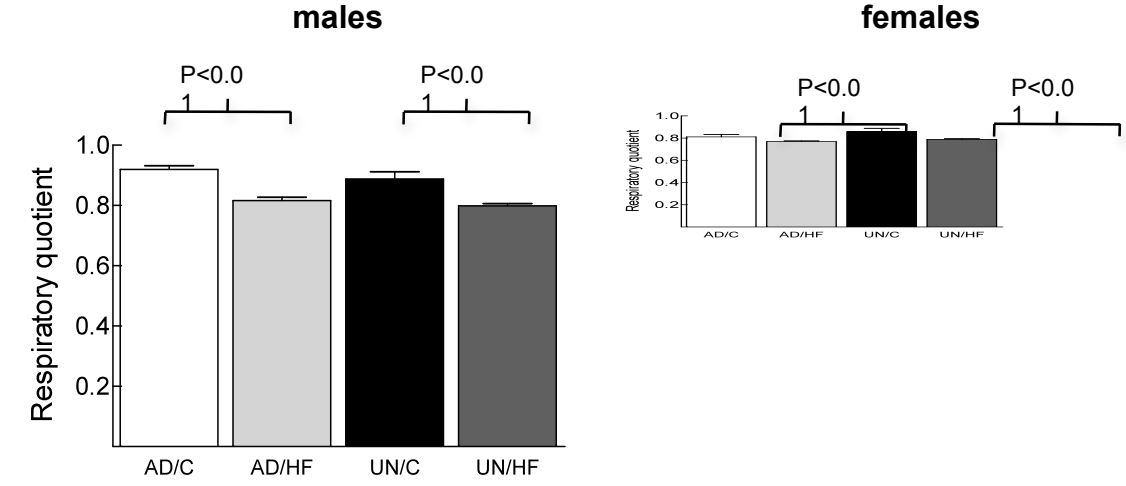


Figure 25. Respiratory quotient in male and female high-fat (HF) or chow (C) fed offspring from dams who were either globally undernourished (UN) or fed ad-libitum (AD) during pregnancy (n=5-8 per treatment group).

3.4.3. Systolic blood pressure

Both male and female offspring from ad-libitum fed dams showed elevated systolic blood pressure ($p<0.01$) following post weaning HF-feeding (Figure 26). There was a further elevation of systolic blood pressure in male and female C-fed offspring from UN dams ($p<0.01$). However post weaning HF feeding did not further increase the elevated blood pressure in offspring from UN dams. Nevertheless, there were no significant differences between UN/C and UN/HF male and female offspring.

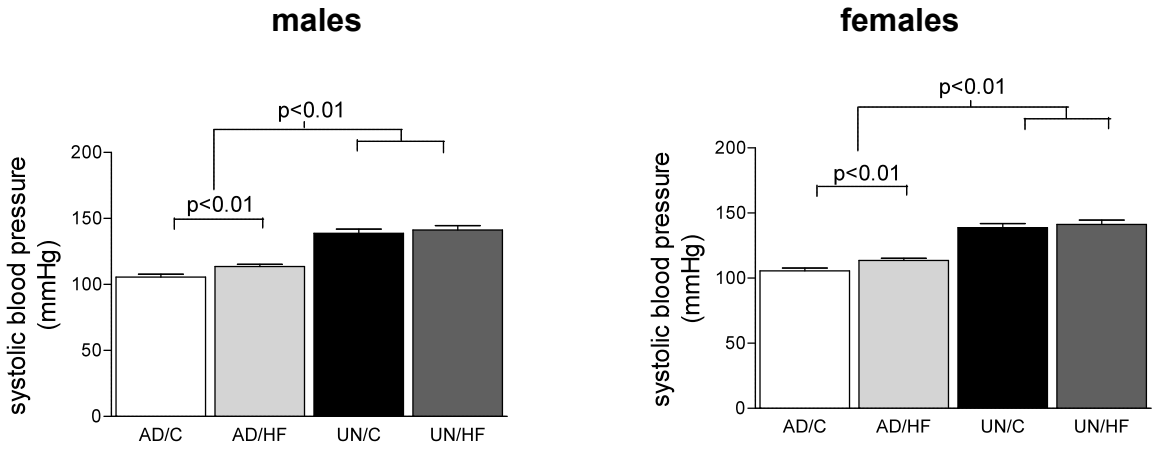


Figure 26. Systolic blood pressure in male and female high-fat (HF) or chow (C) fed offspring from dams who were either undernourished by way of a global food-restriction (UN) or fed ad-libitum (AD) during pregnancy (n=5-8 per treatment group).

3.4.4. Total body fat and individual fat depots

Male and female HF-fed offspring from AD dams exhibited increased adiposity (Figure 27) compared to AD/C offspring ($p<0.01$). Male and female UN/C offspring were also fatter than AD/C offspring ($p<0.05$). HF-fed offspring from UN dams exhibited further increased adiposity over and above the increased adiposity found in the AD/HF male offspring ($p<0.01$) . As one would expect, HF-fed offspring irrespective of maternal diet were fatter than their chow-fed counterparts ($p<0.01$) in both male and female offspring ($p<0.01$).

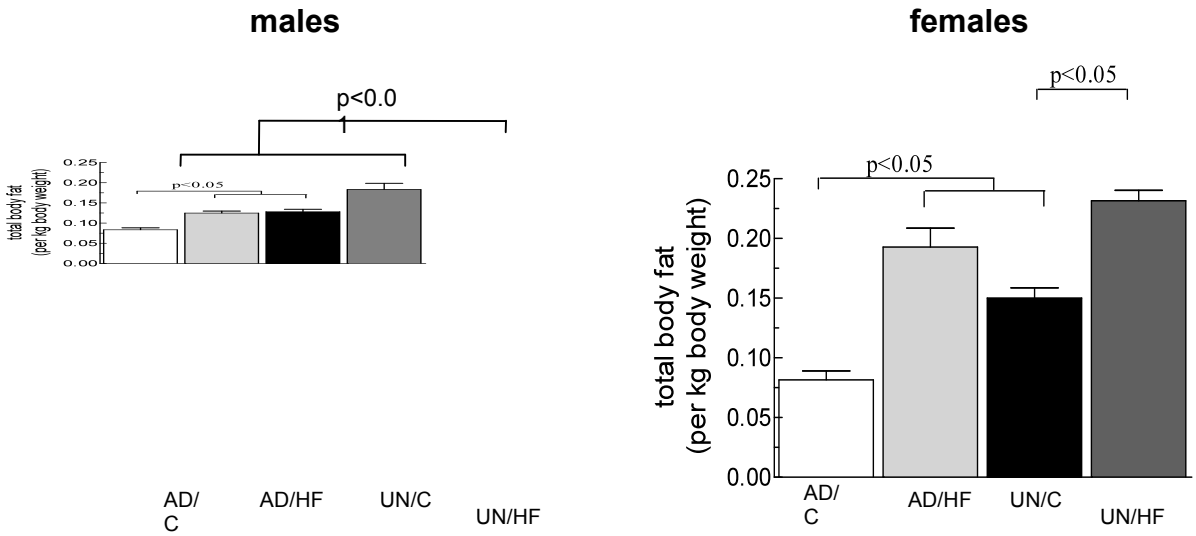


Figure 27. Total body fat (expressed as per kg of body weight) in male and female high-fat fed offspring (HF) and chow-fed offspring (C) from dams who were undernourished (UN) or fed ad-libitum (AD) during pregnancy (n=5-8 per treatment group).

Both UN/HF and AD/HF female offspring exhibited 4- to 5-fold more gonadal fat respectively, compared to chow-fed offspring ($p<0.05$). Male UN/C offspring however, had 90% more gonadal fat than AD/C offspring ($p<0.05$). The female UN/C offspring had twice more inguinal fat than the AD/C animals. Female UN/HF offspring had higher amounts of interscapulary fat compared to AD/HF offspring ($p<0.05$). In males, UN/HF and UN/C had greater amounts of gonadal, inguinal, interscapulary and retro-peritoneal fat

compared to their counterparts that were not undernourished *in utero* ($p<0.05$). UN/C male offspring also had higher levels of perirenal fat compared to AD/C offspring ($p<0.05$).

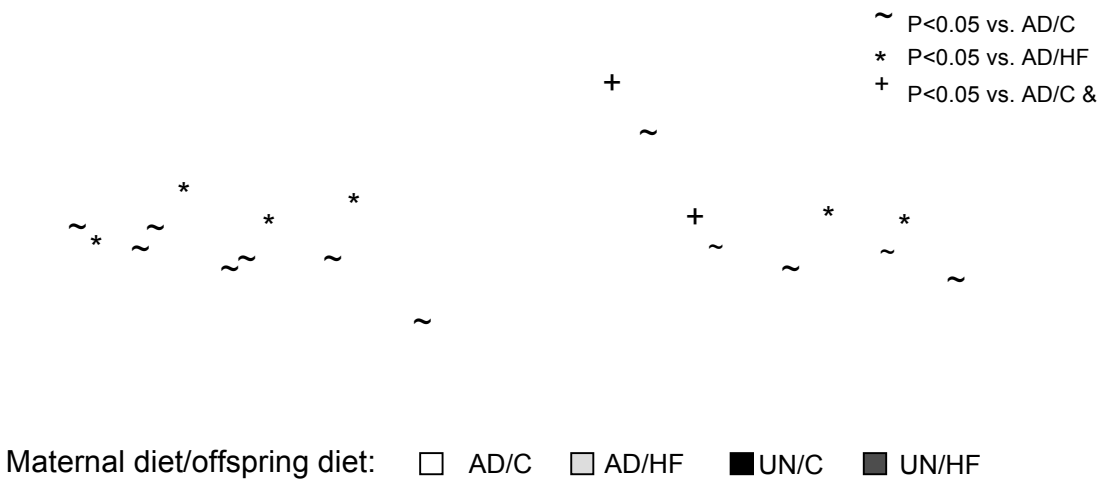


Figure 28. Weight of individual fat depots in male and female high-fat fed offspring (HF) and chow-fed offspring (C) from dams who were undernourished (UN) or fed ad-libitum (AD) during pregnancy (n=5-8 per treatment group).

3.4.5. Blood glucose levels

UN/HF male offspring exhibited elevated plasma glucose levels compared to all other treatment groups ($p<0.05$). In females however, elevated plasma glucose levels were observed in UN/C offspring compared to AD/C offspring ($p<0.05$). Female UN/HF offspring also exhibited elevated plasma glucose levels compared to AD/HF offspring ($p<0.05$). No other significant differences were observed between groups.

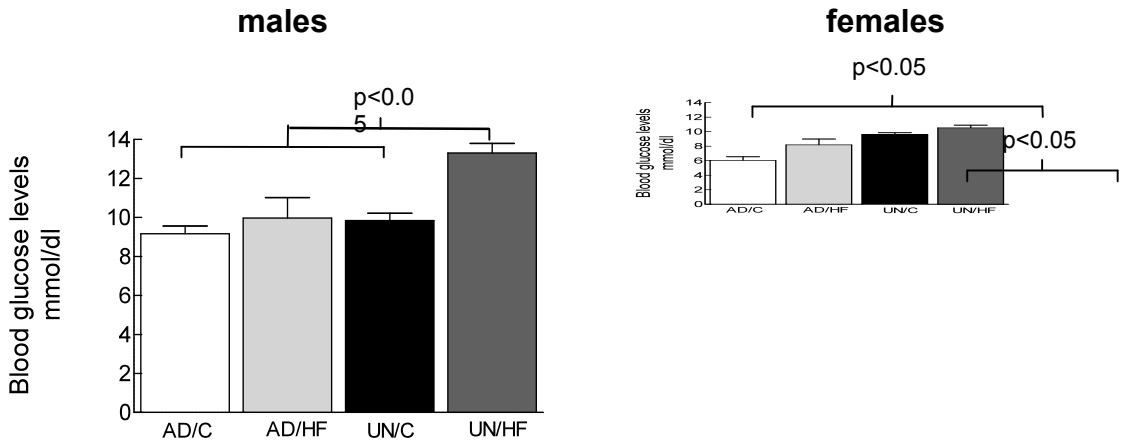


Figure 29. Blood glucose levels in male and female high-fat (HF) or chow (C) fed offspring from dams who were undernourished (UN) or fed ad-libitum (AD) during pregnancy (n=5-8 per treatment group).

3.4.6. Correlation analysis

3.4.6.1. Total body fat and blood glucose levels

There was a significant correlation between total body fat and blood glucose levels in male offspring ($p<0.005$), but not in female offspring (Figure 30). In males, as body weight increases, there is a corresponding increase in blood glucose levels.

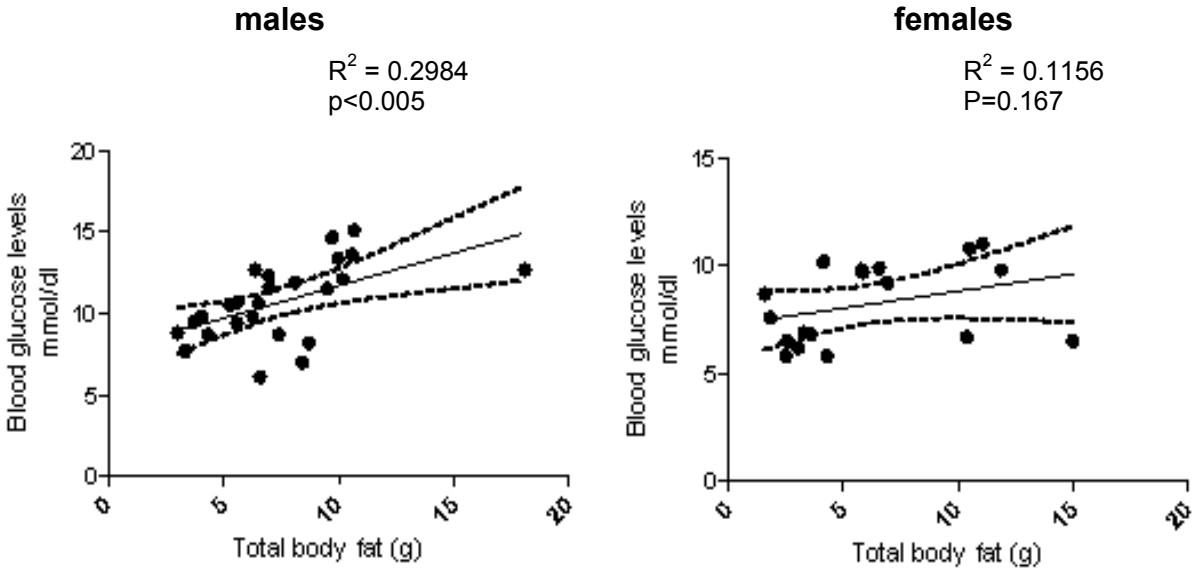


Figure 30. Correlation between total body fat and blood glucose levels in male and female offspring.

3.4.6.2. Total body fat and energy expenditure

There was a significant inverse correlation between total body weight and energy expenditure in male offspring ($p<0.001$), which were not found in females (Figure 31). Thus in males, as total body fat increases, there is corresponding reduction in energy expenditure.

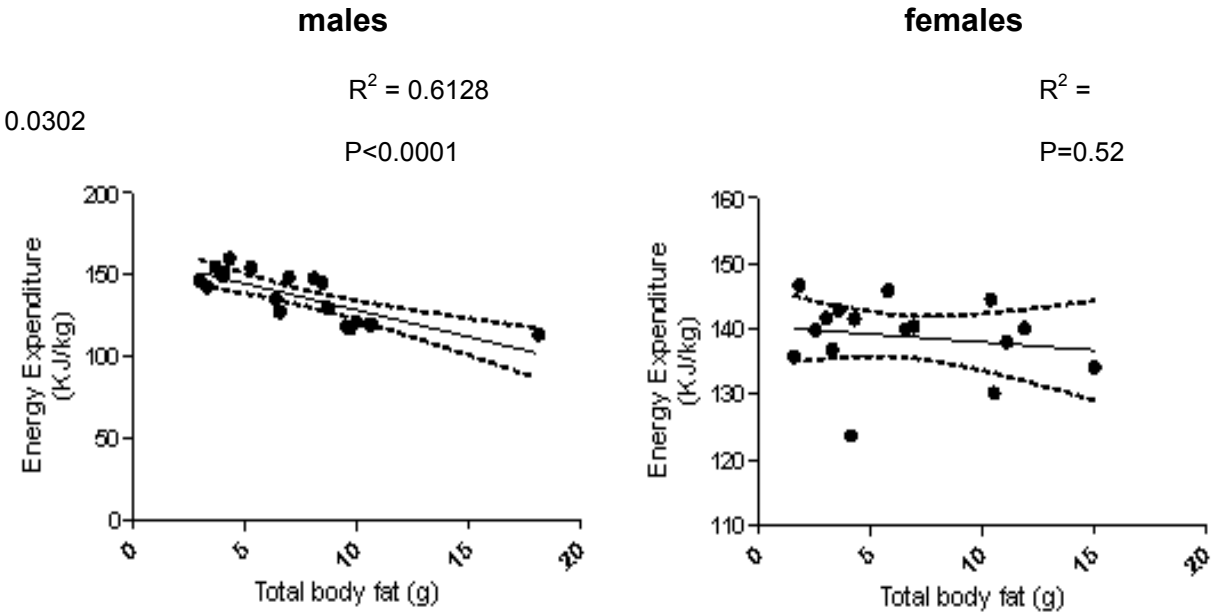


Figure 31. Correlation between total body fat and energy expenditure in male and female offspring.

3.4.7. Phenotypic interactions

There were maternal diet effects (see Table 6) in both male and female offspring for total body fat ($p<0.001$), energy expenditure ($p<0.05$), and systolic blood pressure ($p<0.0001$ in males, 0.001 in females). There was also a significant effect of the post weaning HF offspring diet on total body fat ($p<0.01$) for both sexes and for blood glucose in females ($p<0.01$). We also observed a maternal x offspring dietary interaction ($p<0.001$) for total body fat and energy expenditure but only in males and an interaction in both sexes for blood glucose ($p<0.001$).

Table 6. Results from 2-way ANOVA analyzing maternal x offspring dietary interactions for total body fat (TBF), energy expenditure (EE), systolic blood pressure (SBP) and blood glucose (BG).

	Physiological parameters			
	TBF	**EE**	**SBP**	**BG**
Maternal diet effect				
males	p<0.001	p<0.05	p<0.0001	ns
females	p<0.001	p<0.05	p<0.001	p<0.01
Offspring diet effect				
males	p<0.01	ns	ns	p< 0.01
females	p<0.01	ns	ns	ns
Maternal x offspring diet effect				
males	p<0.001	p<0.001	ns	p<0.001
females	ns	ns	ns	p<0.001

ns = no significant difference.

3.5. Discussion

I have shown that male and female offspring from dams that were undernourished by 50% during pregnancy and fed a chow diet post weaning (UN/C) had increased body weight gain compared to chow-fed offspring from dams that were fed *ad-libitum* during pregnancy (AD/C). Both high fat-fed male and female offspring from undernourished dams (UN/HF) showed similar body weight trajectory compared with high fat-fed offspring from *ad-libitum* fed dams (AD/HF). However a significant catch-up growth was observed in UN/HF male offspring compared to AD/HF group between weeks 6 and 8 of age. Despite the similarity in body weight trajectory between the AD/HF and UN/HF offspring, both male and female UN/HF offspring exhibited greater adiposity than the AD/HF group. In a similar fashion, male and female UN/C offspring also had greater adiposity than the AD/C animals. This suggests that offspring from undernourished dams have become more efficient in storing fat compared to those from *ad-libitum* fed dams irrespective of the offspring's diet.

These results are in agreement with the human scenario during the 1944 Dutch winter famine, where the offspring exposed to *in utero* undernutrition were predisposed to fat accumulation and obesity in later life [15]. This may be due to alterations in their metabolic efficiency in storing energy as fat. The predictive-adaptive response theory [20], suggest that the fetus makes adaptations *in utero* that are in accordance with the predicted postnatal nutritional environment. These metabolic adaptations which promote greater energy storage may not necessarily be beneficial during fetal development, but may provide sufficient survival capabilities, such as allowing for greater fat storage in a nutrient-depleted nutritional environment, if the prediction about the postnatal nutritional environment is correct. If however the actual postnatal environment differs from that which was predicted, catastrophic health problems may occur. This may explain why in parts of Africa, fetal malnutrition is not associated with diabetes and obesity [24], as the

postnatal environment is similar to the prenatal environment. Thus the predicted response is correct and programmed events occur which are appropriate to the nutritional needs of the offspring. The nutritional manipulation in my study produced a moderate pre- and postnatal nutritional mismatch in the UN/C group compared with the UN/HF animals, which is exposed to a more severe form of pre- and postnatal nutritional mismatch. In contrast, the AD/C offspring, which is the only experimental group not exposed to a dietary mismatch, had normal systolic blood pressure and blood glucose levels.

Male UN/HF and UN/C offspring exhibited greater fat accumulation in the various fat depots (except for perirenal fat) compared to AD/HF and AD/C offspring respectively. Both male and female UN/HF groups have greater overall adiposity and reduced energy expenditure compared to the AD/HF, suggesting attenuation in energy expenditure which may in turn be responsible for the increased adiposity observed in these animals. In fact, reduced energy expenditure was strongly correlated with increased adiposity in the male offspring. This may explain the increased adiposity in male UN/C offspring compare to AD/C offspring. This does not however explain the increased adiposity seen in the UN/C females. Moreover, in contrast to male offspring there was no correlation between total body fat and energy expenditure in females. The reason for the increased adiposity in female UN/HF offspring might therefore be influenced by their steroidal milieu, particularly that of estrogen, over substrate oxidation. It has been reported in rats that there is an increase fat accumulation in females despite no observed changes in energy expenditure; this is perhaps due to the known effects of estrogen on localized fat deposition [174].

The finding in this chapter of reduced energy expenditure in male offspring exposed to the greatest degree of nutritional mismatch suggest that predictive adaptive responses by the fetus were inappropriate, resulting in increased susceptibility to an obese phenotype. In

male UN/C offspring, while prenatal nutrition was suboptimal, there is a lesser degree of nutritional mismatch compared with the UN/HF group. This lesser degree of nutritional mismatch resulted in reduced energy expenditure in male UN/C offspring compared to AD/C animals. Energy expenditure, as previously explained, has several components. Adaptive thermogenesis, which in rodents occurs in the intrascapular brown adipose tissue (iBAT), accounts for the majority of individual variation in energy expenditure [110]. The other components, which include obligatory energy expenditure do not vary greatly between individuals and are stable under various conditions. Adaptive thermogenesis on the other hand is the one component of energy expenditure which is under constant regulation depending on physiological state and the energetic requirements of the individual. For example, adaptive thermogenesis increases during cold exposure and HF feeding to aid heat production and limit excess energy storage respectively. This also explains why there is great variation in adaptive thermogenesis between individuals [111]. While previous studies in rodents have shown the importance of iBAT to energy expenditure regulation [106], its influence in humans has been questioned. Moreover, its existence was thought to be limited to infants and newborns [175]. However several recent studies have not only confirmed the presence of iBAT in adult humans, but also showed that it has an active role in energy expenditure [176-178]. Studies have suggested that this is the component of energy expenditure which is impaired following undernutrition *in utero* [97,179]. I also observed reduced respiratory quotient in the HF-fed male and female offspring, irrespective of maternal nutrition during pregnancy. While there were no differences in calorie intake per day between offspring groups, the finding of reduced respiratory quotient in HF-fed offspring indicate that these offspring are consuming significantly greater amounts of fat compared to chow-fed offspring. Dietary fat is the main determinant of respiratory quotient, and its reduction in HF-fed offspring also suggests that these animals are consuming, digesting and utilizing dietary fat [180]. The

fact however that energy expenditure is reduced and total body fat is increased in UN/HF offspring suggests that they are not burning as much fat as they are storing it.

The elevated blood glucose levels in male UN/HF offspring compared to AD/HF offspring most likely reflect the increased adiposity seen in these groups. This is supported by a correlation between total body fat and blood glucose levels in male offspring. While UN/HF female offspring exhibited elevated blood glucose levels compared to AD/HF females, correlation analysis suggests that body fat deposition is not a causal factor for the increase glucose levels. This is in agreement with a previous study in rats demonstrating that while fat-induced insulin resistance is common in male rats, it does not occur in female, which appear to be resistant to the negative effects of various bi-products of lipid metabolism on insulin-sensitivity such as ceramide [181]. Thus, mechanisms other than fat accumulation might be responsible for the elevated blood glucose levels observed in female UN/HF offspring. A number of studies in humans have demonstrated, albeit without a hypercaloric postnatal diet, that a nutritional mismatch between prenatal and postnatal diet results in insulin resistance [179,182]. In female UN/C offspring, but not males there was an increase in blood glucose levels compared to C/C offspring. This is in agreement with a previous finding in rats, demonstrating elevated fasting blood glucose levels and impaired glucose tolerance in female but not in male offspring that were growth-restricted *in utero* by uterine artery ligation [183]. Thus these results indicate a possible insulin-resistance and impaired glucose tolerance in offspring exposed to the greatest degree of nutritional mismatch.

The results in this chapter also showed that a post weaning HF diet induces elevated systolic blood pressure in offspring from *ad libitum*-fed dams. There was a further increase in systolic blood pressure in male and female chow-fed offspring from undernourished dams during pregnancy. There was however no further increase in systolic blood pressure

in the UN/HF offspring. This finding of an increased systolic blood pressure in UN/C is consistent with other studies, in which similar levels of global undernutrition was imposed during pregnancy [184,185]. However it is only in the current study where the effects of changing the post weaning diet were examined. I have found that giving previously undernourished offspring a HF diet post weaning does not result in further elevations of systolic blood pressure from that seen in UN/C offspring. One study has demonstrated that there are no further changes in plasma cholesterol levels when a HF diet is given to offspring exposed *in utero* to global undernutrition [186]. Given that plasma cholesterol is strongly associated with blood pressure and could predict hypertension in rats [187], this might explain the lack of changes in systolic blood pressure in UN/HF offspring compared to UN/C offspring.

The results in this chapter suggest an increased risk of developing metabolic disturbances lead to obesity in the offspring following *in utero* exposure to maternal undernutrition. Many adverse effects of global undernutrition during pregnancy, particularly in males are observed in the absence of a subsequent post weaning high fat feeding, suggesting that a lesser degree of nutritional mismatch is sufficient to produce altered metabolic and cardiovascular outcomes.

The use of a global caloric restriction during pregnancy as explained in Chapter 1 is one of two major forms of dietary manipulation used to represent maternal undernutrition. The other is protein restriction, in which the content of the macronutrient protein in the diet is reduced, while the amount of calories in the diet and the amount of diet available to the animals is not different to that of control animals. The succeeding chapter examines the effect of this protein restricted diet during pregnancy on the metabolic and cardiovascular phenotype of the offspring.

Chapter 4

The effects of maternal protein-restriction during pregnancy and a post weaning high-fat diet on metabolic and cardiovascular phenotype of the offspring

4.1 Introduction

A number of human studies have demonstrated that nutrient imbalance in the form of a protein deficiency is a particularly common cause of mortality in the developing world [188,189]. Protein deficiency, when manifested in early development, results in metabolic abnormalities characteristic of diseases such as Kwashiorkor and Marasmus [190]. Thus in this chapter and in succeeding chapters, I looked at manipulating the diet of the pregnant mouse dams by reducing the protein content of their diet instead of reducing their total food intake. One of the main reasons for doing protein-restriction during pregnancy, as opposed to a global food restriction, was to determine if by reducing specific macronutrients without altering the caloric content of the diet, one will find these offspring to have a different phenotypic outcome to those from dams that were subjected to caloric restriction by global undernutrition. Another reason for using a protein-restricted maternal diet was because of the difficulty in maintaining offspring viability during pregnancy and incidence of cannibalism by the dams on their newborns following a 50% reduction in the dam's food intake during pregnancy. There is also an advantage of being able to extend the maternal dietary manipulation to include the lactation period. In the next chapter (Chapter 5), I attempted to dissect and examine the importance of the maternal nutritional status during lactation by comparing maternal dietary manipulation exclusively during pregnancy to one that was imposed during both pregnancy and lactation periods.

A majority of animal studies that have used maternal protein restriction paradigm have reduced the protein content of the diet by 50% during the pregnancy period. This has been shown to bring about growth-restriction to the developing fetus resulting in hypertension, vascular impairment and cardiovascular dysfunction, which are characteristic of the metabolic syndrome [191, 192]. However, it remains to be determined whether the effects of maternal protein restriction during pregnancy on the phenotypic outcomes of their offspring can be exacerbated by mismatched offspring diet containing a high percentage of

fat. This mismatch between prenatal and post weaning nutrition mimics the nutritional paradigm that is prevalent in countries experiencing socio-economic transition and the dietary changes that accompanies it. A majority of the human population in these countries, including China, India and those in South America, which have previously experienced food scarcity and poor nutrition are now going through a period of overabundance in food supply to the extent that there is now excessive consumption, particularly of foodstuffs that are high in fat and calories. What is extraordinary is that this phenomenon has occurred very rapidly within a single generation. There is now increasing incidence of obesity, cardiovascular disease and metabolic diseases such as type-2 diabetes in these countries [193] and this is predicted to rise even further in the future [193].

In animal studies, it has now been demonstrated that a hypercaloric postnatal diet following *in utero* exposure to maternal undernutrition is important in the development of obesity [26]. The importance of a postnatal hypercaloric diet is probably a reflection of two factors. Firstly, the 'mismatch' between the pre and postnatal nutritional status is likely to have pathological consequences due to an inappropriate adaptation to the prenatal insult. Secondly, various organ systems in rodents are still developing even after birth. The rodent brain is a very good example of organ plasticity occurring even after birth. It has been reported that the neuronal network involved in appetite regulation within the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the rodent hypothalamus is not establish until at least 15 days after birth [194, 195] despite the fact the genes involved in appetite regulation are already evident as early as embryonic day 12 [196]. The early postnatal period therefore provides an important opportunity for programming to take place because a lot of developmental plasticity takes place during this period, in addition to those occurring during the prenatal period. Studies in rodents emphasize postnatal nutrition in the programming of appetite [26]. It has previously been reported that there is increased weight gain, hyperphagia, hyperleptinaemia, hyperglycaemia and insulin resistance in rats

overnourished during lactation [197], and this might be due to mechanisms such as neuronal rewiring during development [198].

Going back to the effect of maternal protein restriction during pregnancy, it has been shown to result in fetal growth retardation leading to low birthweight, hypertension and metabolic abnormalities in adulthood even when these offspring are on a normal chow diet [199]. Moreover, these offspring exhibited abnormal neuronal differentiation, including decreased NPY expressing neurons, which may explain the observed decrease in their food intake or hypophagia [200]. It remains to be determined however what the effects are of catch-up growth on these offspring by post weaning feeding with a diet that is high in fat and calories, similar to the human scenario of population in transition that I have alluded to earlier in the discussion.

Since obesity is a consequence of increased food intake and/or reduction in energy expenditure, I therefore assessed the effect of the pre- and postnatal dietary mismatch on the expression of genes that regulate appetite in the hypothalamic region of the offspring brain and of genes that are involved in thermogenesis in the brown adipose tissues. Our group has previously reported that genes involved in appetite regulation, such as NPY, POMC and the leptin receptor Ob-Rb, are already expressed as early as embryonic day 12 in the fetal brain and that maternal dietary protein restriction can alter their mRNA level of expression [196]. It remains to be determined however whether these changes in gene expression due to maternal dietary protein restriction during pregnancy is still evident in the adult offspring and whether post weaning high fat feeding will result in a different phenotypic outcome in these offspring.

Several studies in rodents have also suggested the possible involvement of increased energy efficiency in the accelerated postnatal fat deposition in offspring from

undernourished dams during pregnancy. One study has demonstrated greater body weight gain in rat offspring from dams that were malnourished during pregnancy and were themselves weaned onto a hypercaloric diet [26]. This increased weight gain exceeded what could be attributed to the observed hyperphagia in these offspring, suggesting that these animals have become more energy efficient. Several other studies have supported these results [201, 202]. Given that reduced thermogenesis is a key mediator of energy expenditure [203], and that reduced thermogenesis has been linked to negative effects on metabolism [204], it is possible that reduced thermogenesis plays an important role during the catch-up growth period to alter metabolism and predisposes an individual to becoming obese. β3-adrenergic receptor and UCP-1 are key components of adaptive thermogenesis. They are expressed in the intrascapular brown adipose tissue (iBAT), which is the primary tissue for adaptive thermogenesis in mice and therefore most consequential to affecting their total daily energy expenditure. The presence of brown adipose tissue (BAT) in adult humans has long been considered controversial. However, several recent studies have confirmed not only its presence in adult humans, but that it has an active role in adaptive thermogenesis similar to what is observed in mice [176-178]. The activity of UCP-1 in the BAT is under the control of the sympathetic nervous system, mediated by the catecholamines [205]. UCP-1 is activated by the β-3 adrenergic receptor. Its activity can be activated by noradrenaline, and this in turn increases the expression of UCP-1 in BAT [206]. It has been shown that mice lacking β3-adrenergic receptors are obese and unable to increase heat production is response to cold exposure [207]. Nevertheless, it remains to be determined what are the effects of maternal dietary protein restriction during pregnancy on the expression of the β3-adrenergic and UCP-1 in the iBAT and the extent by which feeding a high-fat diet post weaning impact on the expression pattern of these genes in the adult offspring. This is interesting in light of recent work suggesting a possible impairment of adaptive thermogenesis associated with catch-up growth following fetal undernutrition [179].

4.2. Aims

1) To determine the metabolic and cardiovascular phenotype of adult offspring from protein restricted dams during pregnancy.

2) To examine whether there are changes in the expression of genes involved with appetite regulation in the hypothalamus and those involved in thermogenesis in the iBAT of adult offspring from dams that were fed a protein-restricted diet during pregnancy.

3) To look at the effect of post weaning high-fat feeding on the cardiovascular and metabolic phenotypic outcomes and gene expression patterns in the adult offspring.

4) To find out whether these changes are sex-specific.

4.3. Methods

4.3.1. Experimental procedures

All procedures were carried out in accordance with the UK Animal Scientific Procedures Act of 1986. Female MF-1 mice were individually housed under a 12h light-dark cycle and given full access to drinking water throughout the study. These females were time-mated at 8-10 weeks of age and on confirmation of pregnancy were randomly divided into two dietary groups (see Figure 32). One group (n=10) was fed a standard chow (C) diet containing 18% casein and the other group (n=10) a protein-restricted (PR) diet containing 9% casein. These diets were formulated so that they remained isocaloric (see General Methodology in Chapter 2 for details of the C and PR diet). Pregnant dams were fed their respective diets throughout gestation. At birth litter size was standardized to 8 pups per litter. From birth to the time of weaning all lactating dams were fed the C diet. From weaning to the end of the study when the offspring are at 32 weeks of age, they were either fed the C or a high fat (HF) diet (see General Methodology in Chapter 2 for details of the HF diet).

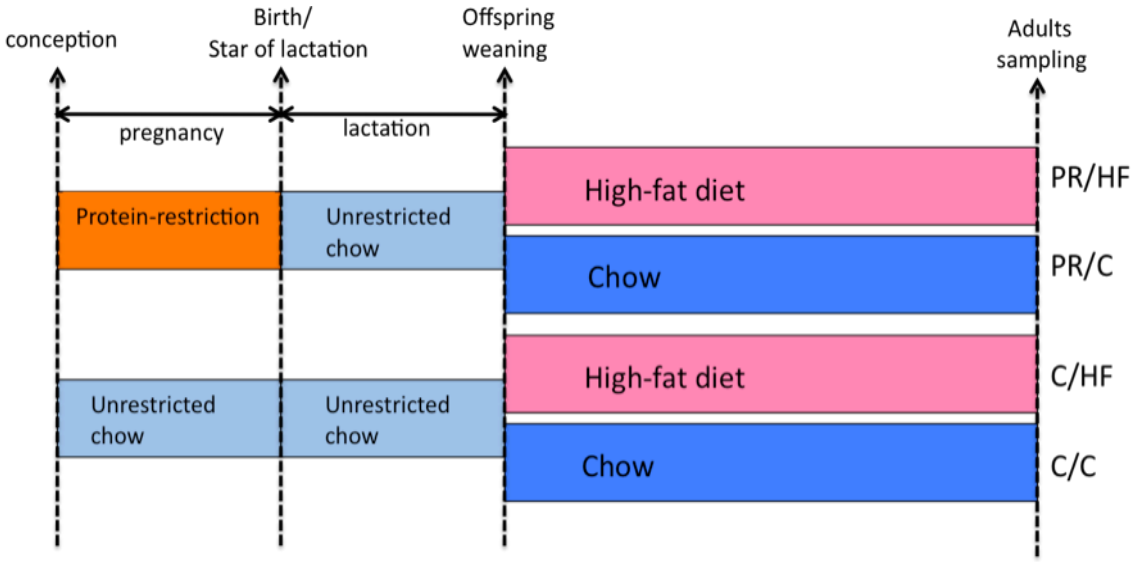


Figure 32. Experimental protocol. Pregnant dams were fed a protein restricted (PR) diet or standard chow (C) diet during pregnancy and both groups fed the C diet during the lactation period. Offspring from both sets of dams were weaned onto a high fat (HF) diet (generating dam/offspring dietary groups PR/HF and PR/C), or the C diet (generating dam/offspring dietary groups C/C and C/HF).

Food intake and body weight of the offspring were monitored during the course of the study. Between 28-30 weeks of age the offspring's locomotor behaviour (see below) and blood pressure were measured (see General Methodology in Chapter 2 for details). Adult offspring were humanely killed at 32 weeks of age by CO_2 inhalation and cervical dislocation. Each offspring group had a sample size of between 7-11 animals. Blood was collected by cardiac puncture and the heart and various fat depots dissected and weighed (see General Methodology in Chapter 2 for details). Cumulative fat depot weights for each animal were compared with total body weights and body fat as a percentage of total body weight was calculated. Hypothalamic and cortical tissue blocks were also collected (see General Methodology in Chapter 2 for sampling details), snap frozen in liquid nitrogen and stored at -80°C for future processing.

4.3.2. Analysis of locomotor behaviour

In order to determine locomotor behaviour, offspring were subjected to an open field activity test as previously described [208]. The test comprised 3 minute observations in an open field (white PVC arena, 30 cm^2) with automated recording of total distance travelled, number of hind rears (vertical count), jumps, time spent resting and average velocity.

4.3.3. Plasma leptin measurement

Plasma leptin levels were determined using a mouse leptin radioimmunoassay kit. The assay was kindly performed by Prof Mohammed Ghatei at Imperial College in London. The assay is based on a polyclonal antibody raised against recombinant rat leptin in guinea pigs and on calibrators and ^{125}I-labeled tracer prepared from recombinant rat leptin. Calibrators (0.5, 1, 2, 5, 10, 20, and 50 μg/L) or specimens were pipetted in duplicate into tubes at 100 μL each and mixed with anti-leptin antibody (100 μL). After incubation for 18–24 h at room temperature (disequilibrium assay format), 100 μL of ^{125}I-tracer was added to each tube, and incubation continued for another 18–24 h. Cold precipitating antibody (1.0 mL; anti-guinea pig IgG, raised in goats) was added to all tubes and incubated for 20 min at 4°C to precipitate the antibody/leptin complex. Centrifugation for 20 min at 2500*g* at 4°C yielded visible pellets; the supernatants were decanted, and the radioactivity in the pellets was counted. Log values of calibrators were plotted vs the calibrator-bound counts/zero calibrator-bound counts (*B*/*B*o) to generate a curve for calculation of unknowns.

4.3.4. Gene expression analysis

4.3.4.1. Validation of internal standards for real-time PCR

The validity of results obtained using reverse transcription-polymerase chain reaction (RT-PCR) to measure gene expression is dependent on accurate data normalization. The most common method for normalization is the use of internal control reference genes, often referred to as 'housekeeping' genes (HKG). However, it has been reported that while the expression of some HKGs are constant under certain conditions, it can change significantly in others [209-212]. This has been shown even in cases in which commonly accepted HKGs, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and 18s ribosomal RNA (18s rRNA), were used as internal controls [213-215]. Thus, it is necessary to characterize the suitability of various HKGs to serve as internal RNA controls under particular experimental conditions where transcription effects are being investigated.

In gene expression studies in the rat brain, GAPDH and β-actin were found to be stable in the cortex and hippocampus in animals subjected to experimental paradigms such as aging, dietary restriction and dexamethasone treatment [216]. However, the stability of these HKGs in the hypothalamus has not been examined in animals subjected to nutritional manipulation during development and in postnatal life. The expression of some genes within this region of the brain are known to be sensitive to conditions in which the *in utero* nutritional environment is suboptimal [217,218] and is exacerbated when the post weaning diet is rich in fat [219]. I therefore looked into the expression levels of the housekeeping genes GAPDH, β-actin and 18s rRNA in the hypothalamic region of the brain of offspring subjected to a nutritional mismatch, wherein their mothers were given a protein-restricted diet throughout pregnancy and weaned offspring fed a HF diet to adulthood.

Total RNA was extracted from the hypothalamic and cerebral cortex tissue blocks. The cerebral cortex tissues were used as control tissue samples. Tissue RNA was reversed

transcribed. Specific primers and probes for β-actin and GAPDH were designed based on their published sequences using the Primer Express™ (v1.0) software. The β-actin and GAPDH primer and probe oligonucleotide sequences are shown in Tables 7 and 8 respectively.

Table 7. β-actin primers and probe sequences

Forward (sense) Primer	5'-CGT GAA AAG ATG ACC CAG ATC A-3'
Reverse (anti-sense) Primer	5'-CAC AGC CTG GAT GGC TAC GT-3'
Probe	5'-FAM-TTT GAG ACC TTC AAC ACC CCA GCC AT-TAMRA-3'
GenBank accession no.	NM007393.2

Table 8. GAPDH primers & probe sequences

Forward (sense) Primer	5'-TGT GTC CGT CGT GGA TCT GA-3'
Reverse (anti-sense) Primer	5'-CAC CAC CTT CTT GAT GTC ATC ATA C-3'
Probe	5'-FAM-TGC CGC CTG GAG AAA CCT GCC-TAMRA-3'
GenBank accession no.	NM008084

The probe and primers used for 18S ribosomal RNA was commercially purchased (Applied Biosystems, Warrington, UK). Gene transcript levels were then measured by RT-PCR (see General Methodology in Chapter 2 for details of protocol).

4.3.4.2. Hypothalamic gene expression analysis for NPY, POMC and Ob-Rb

Total RNA was extracted from the hypothalamic tissue samples and cDNA was synthesized. Specific primers and probe for NPY, POMC and Ob-Rb were designed based on their published sequences (from PUBMED) using the primer express™ (v1.0) software. Oligonucleotide sequences were synthesized by Eurogentec Ltd (Romsey UK). The primer and probe oligonucleotide sequences for NPY, POMC and Ob-Rb are shown in Tables 9, 10 and 11 respectively.

Table 9. NPY primers & probe sequences

Forward (sense) Primer	5'-GCA GAG GAC ATG GCC AGA TAC-3'
Reverse (anti-sense) Primer	5'-TGG ATC TCT TGC CAT ATC TCT GTC T-3'
Probe	5'-FAM-CGC TCT GCG ACA CTA CAT CAA TCT CAT CA-TAMRA-3'
GenBank accession no.	NM023456

Table 10. POMC primers & probe sequences

Forward (sense) Primer	5'-CTC CTG CTT CAG ACC TCC ATA GA-3'
Reverse (anti-sense) Primer	5'-GGA TGC AAG CCA GCA GGT T-3'
Probe	5'-FAM-AGA GCA GCC AGT GCC AGG ACC TCA C-TAMRA-3'
GenBank accession no.	NM008895

Table 11. OB-Rb primers & probe sequences

Forward (sense) Primer	5'-TGA ATT TCC AAA AGC CTG AAA CA-3'
Reverse (anti-sense) Primer	5'-CCA GAA GAA GAG GAC CAA ATA TCA C-3'
Probe	5'-FAM-TTG AGC ATC TTT TTA CCA AGC ATG CAG AAT C-TAMRA-3'
GenBank accession no.	U46135

Gene transcript levels were then measured by RT-PCR (see General Methodology in Chapter 2 for details of protocol). β-actin was used to normalize expression levels of mRNA for NPY, POMC, and OB-Rb based on initial validation studies in Section 4.3.4.1.

4.3.4.3. Gene expression analysis for UCP-1 and β-3 adrenergic receptor in the interscapulary brown adipose tissue (iBAT)

Gene expression analysis for UCP-1 and β-3 adrenergic receptor in the iBAT was undertaken using the MT-qc PCR (see General Methodology in Chapter 2 for details of protocol). The MT-qc PCR protocol requires standard primers, the first part of which are identical in sequence to the cDNA primers and has a specialist sequence tagged to their

ends. Primer sequences for UCP-1 and β3-adrenergic receptor are shown in Tables 12 and 13.

Table 12. UCP-1 Primer sequences (standard & cDNA)

Forward cDNA Primer	CTT CTC AGC CGG AGT TTC AG
Reverse cDNA Primer	GTC GTA GAG GCC AAT CCT GA
Forward sDNA Primer	CTT CTC AGC CGG AGT TTC AGG TCT ATG AGT CAC AGT ACA TAG
Reverse sDNA Primer.	GTC GTA GAG GCC AAT CCT AAT CCT GAT GGC AAC GTT ACT ATT AAC GGA

Table 13. β-3 adrenergic receptor Primer sequences (standard & cDNA)

Forward cDNA Primer	GAC AGC CTC AAA TGC ATC CT
Reverse cDNA Primer	AGT CTG TCA GCT TCC CTC CA
Forward sDNA Primer	GAC AGC CTC AAA TGC ATC CTG TCT ATG AGT CAC AGT ACA TAG
Reverse sDNA Primer.	AGT CTG TCA GCT TCC CTC CTC CAT GGC AAC GTT ACT ATT AAC GGA

Standard DNA (sDNA) of known concentrations was used to ascertain the concentration of target DNA (cDNA). This was achieved using the Phoretix software program that established the cDNA:sDNA ratio from a computerized image of an agarose gel.

4.3.5. Statistical analysis

All values are presented as mean ± SEM. Effect size estimates for body weight gain and energy intake are from a mixed model analysis [161] that considers all time points through the study, controlling for the set of dam-pup relationships. All other data were analyzed statistically using analysis of variance (ANOVA) followed by the Tukey-Kramer test for comparisons where appropriate. Statistical significance was assumed if $P<0.05$. Certain phenotypic parameters (i.e. blood glucose, systolic blood pressure and total body fat) as well as gene expression of UCP1 and β-3 adrenergic receptor in the iBAT were analyzed by two-way ANOVA for programming (maternal protein-restriction) x HF dietary (post

weaning) interaction. Mixed-model analysis and two-way ANOVA was done with SPSS 14.0 (SPSS Inc). All other analysis was done using Sigma Stat (Systat Software Inc) or Prism (GraphPad Software Inc) statistical programs.

4.4. Results

4.4.1. Food intake & body weight

Similar growth patterns were observed in the C/C, C/HF, PR/C and PR/HF groups, although the HF offspring were 15% heavier than the C groups (Figure 33 and Table 14). This difference in growth trajectory was evident from 5 weeks of age in males (Figure 33), while in females the trend was observed from 12 weeks of age (Figure 33). In PR/HF males, but not in females, energy intake (kcal/day) was reduced by as much as 20% compared to the PR/C group ($p<0.001$, see Figure 33. and Table 15). This difference was observed from 5 weeks of age and maintained up until the termination of the study at 16 weeks of age.

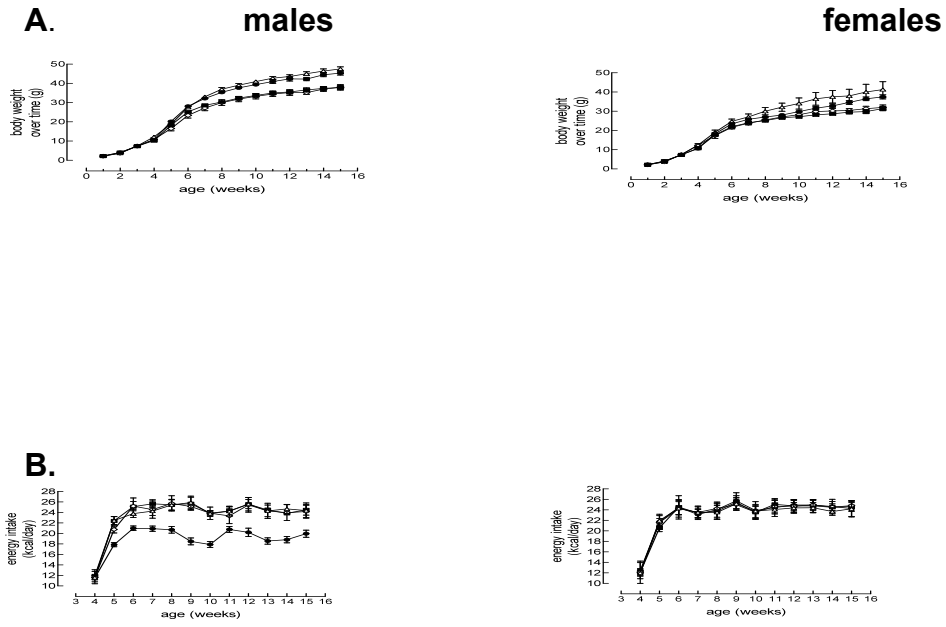


Figure 33. (A) Body weight gain and (B) food intake (kcal/day) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

Table 14 shows the results of mixed model analysis of food intake and body weight, taking into account dam-pup relationships. The table shows the significant reduction in food intake in PR/HF male, but not female offspring. This was the only significant difference in food intake between groups in either males or females.

Table 14. Estimate of mean difference and 95%CI of body weight and energy intake in the offspring

Group comparisons		Variables			
		body weight, g		energy intake, kcal/day	
		males	females	males	females
PR/HF vs PR/C	Mean	1.08	0.51	1.3	0.09
	95% CI	0.3, 1.85	0.41, 1.43	0.78, 1.82	-0.63, 0.83
	P value	<0.001	ns	<0.001	ns
PR/HF vs C/HF	Mean	0.28	0.67	1.25	0.04
	95% CI	-0.49, 1.05	0.3, 1.64	0.72, 1.77	-0.74, 0.83
	P value	ns	ns	<0.001	ns
PR/C vs C/C	Mean	0.02	0.06	0.06	0.25
	95% CI	-0.79, 0.84	-0.92, 1.04	-0.61, 0.5	-1.04, 0.54
	P value	ns	ns	ns	ns

Effect estimates (n = 7-11 per group) are from a mixed model analysis that considers all time points through the study, controlling for the set of dam-pup relationships. ns = no significant difference.

4.4.2. Total body fat and individual fat depots

Total body fat was 50% higher in HF-fed male and female offspring compared to their chow-fed counterparts (Figure 34), regardless of whether their mothers were fed a protein-restricted or chow diet during pregnancy ($p<0.001$).

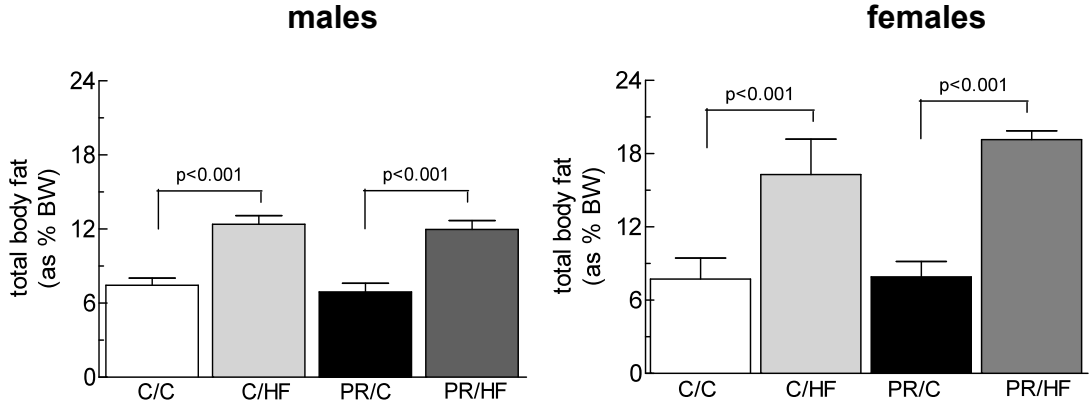


Figure 34. Total body fat (expressed as per kg of body weight) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

In terms of individual fat depot (Figure 35), HF-fed male and female offspring showed between 30% and 120% higher levels of gonadal, inguinal and retro-peritoneal fat compared to chow-fed offspring ($p<0.01$) irrespective of maternal diet.

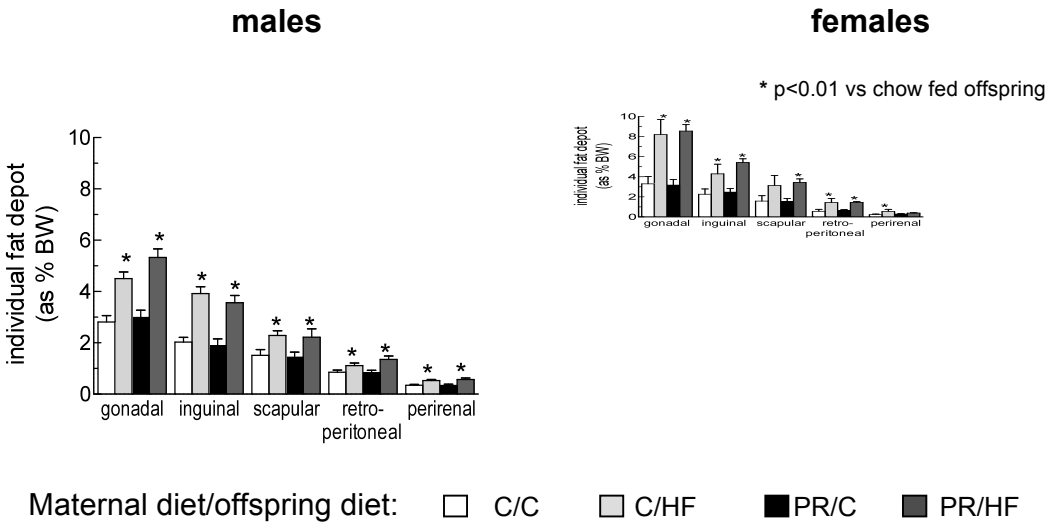


Figure 35. Weight of individual fat depots in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

4.4.3. Plasma leptin levels

Post weaning HF feeding resulted in a two-fold increase in plasma leptin levels ($p<0.001$) regardless of the sex of the offspring or whether they came from PR or C dams (Figure 36). In C-fed female offspring, those that came from PR dams have higher leptin levels ($p<0.05$) compared to the C/C females.

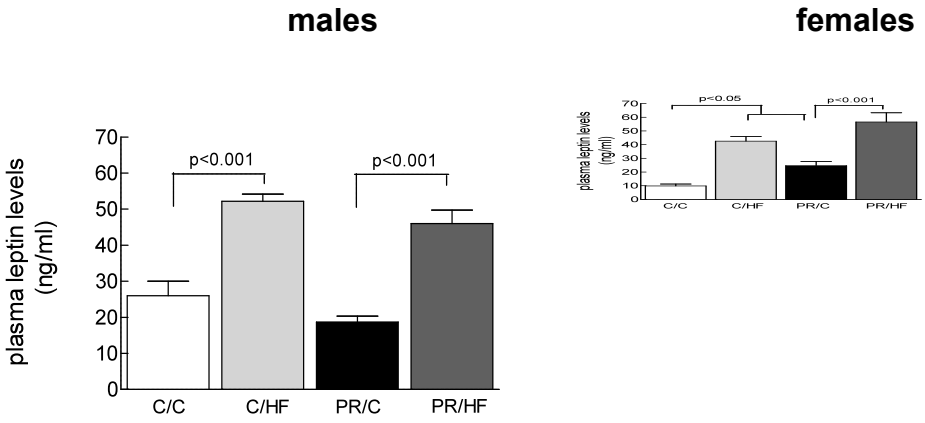


Figure 36. Plasma leptin levels in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

4.4.4. Blood glucose levels

Blood glucose levels were raised by 15% in HF-fed male and female offspring ($p<0.01$), compared to chow-fed offspring, regardless of maternal diet during pregnancy (Figure 37).

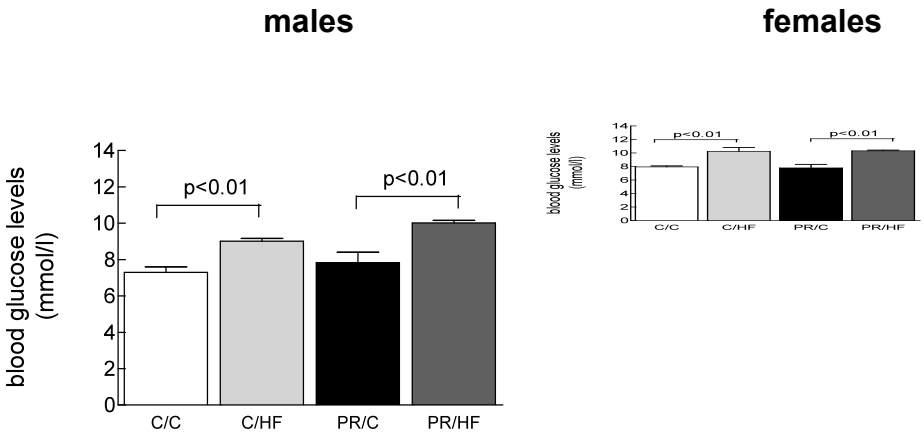


Figure 37. Blood glucose levels in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

4.4.5. Systolic blood pressure

There was a significant elevation of systolic blood pressure in PR/C male ($p<0.001$) and female ($p<0.05$) offspring compared to both C/C and C/HF offspring (Figure 38). Systolic blood pressure was further elevated by 40% in the PR/HF male group ($p<0.05$) and by 45% in the PR/HF female group ($p<0.05$) compared with the PR/C groups, respectively.

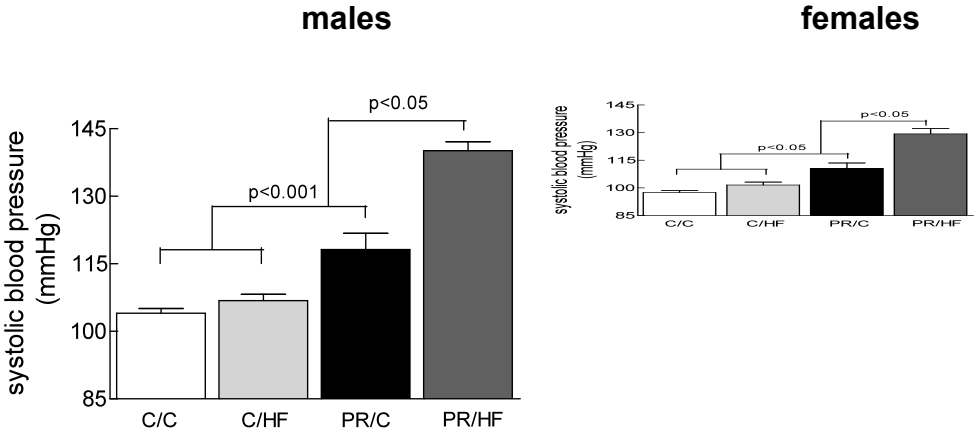


Figure 38. Systolic blood pressure in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

4.4.6. Heart weights

There was a 20% reduction in heart weight (expressed as % body weight) in PR/HF male ($p<0.05$) and female ($p<0.001$) offspring compared to the C/C, C/HF and PR/C offspring (Figure 39).

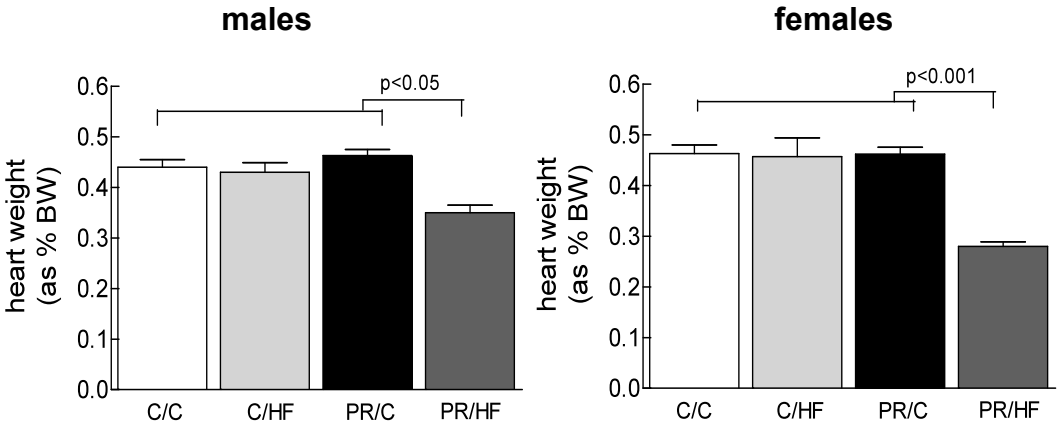


Figure 39. Heart weight (expressed as % body weight) in offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

4.4.7. Locomotor Behaviour

Of the five parameters of locomotor behaviour measured in this study (Figure 40 and 41), significant differences between experimental groups were only observed in the number of jumps (see Figure 41A). In males, the C/HF offspring showed reduced number of jumps compared with their chow-fed counterparts ($p<0.05$). There was also a trend for a reduced number of jumps in the female C/HF group. However this was not found to be significant. However, the female PR/HF group had reduced number of jumps compared to the PR/C group ($p<0.05$).

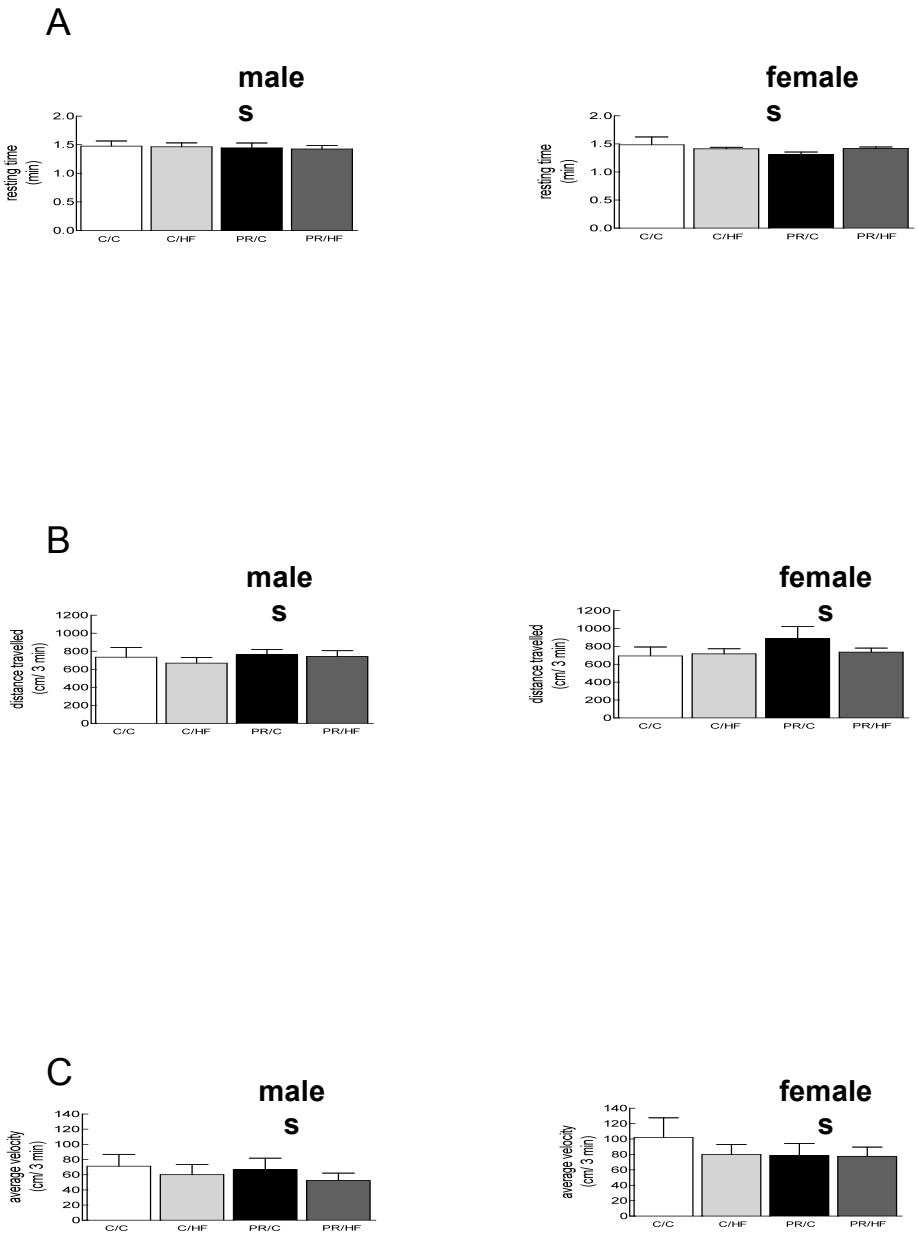


Figure 40. Open field behaviour in terms of (A) resting time, (B) distance travelled and (C) average velocity in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group).

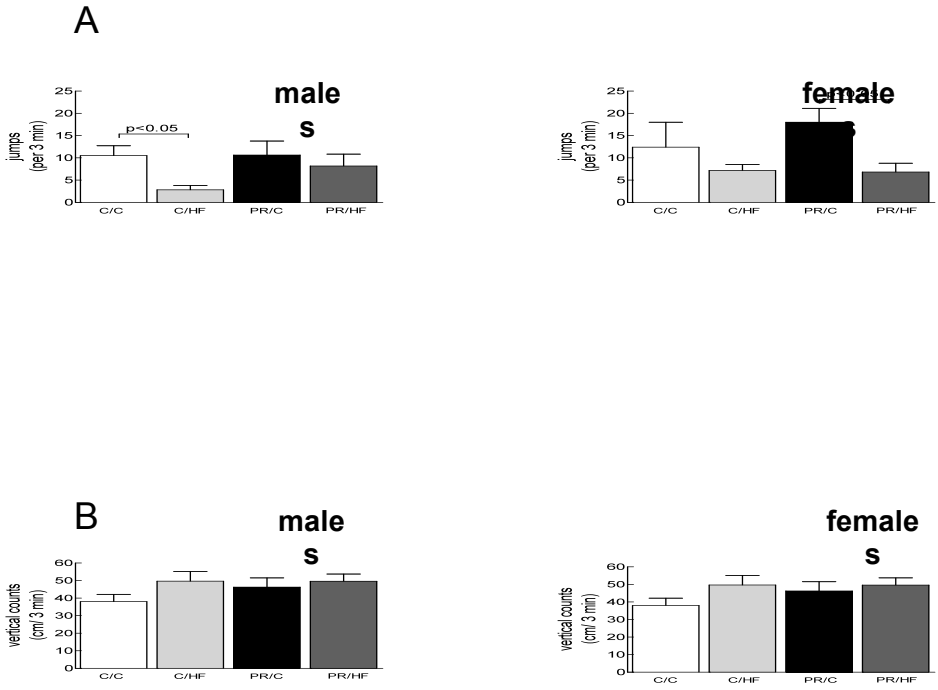


Figure 41. Jumps (A) and vertical counts (B) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group).

4.4.8. Gene expression analysis

4.4.8.1. Validation of GAPDH, β-actin and 18S rRNA as internal standards

Hypothalamic expression of GAPDH in the PR/HF male offspring was elevated by 3-fold ($p<0.001$) compared to the PR/C, C/C and C/HF groups (Figure 42). A similar trend was observed in females, with the PR/HF group demonstrating a 2-fold elevation in GAPDH levels although this was not found to be significant. In the cerebral cortex GAPDH was also found to be stable across the experimental groups and in both sexes.

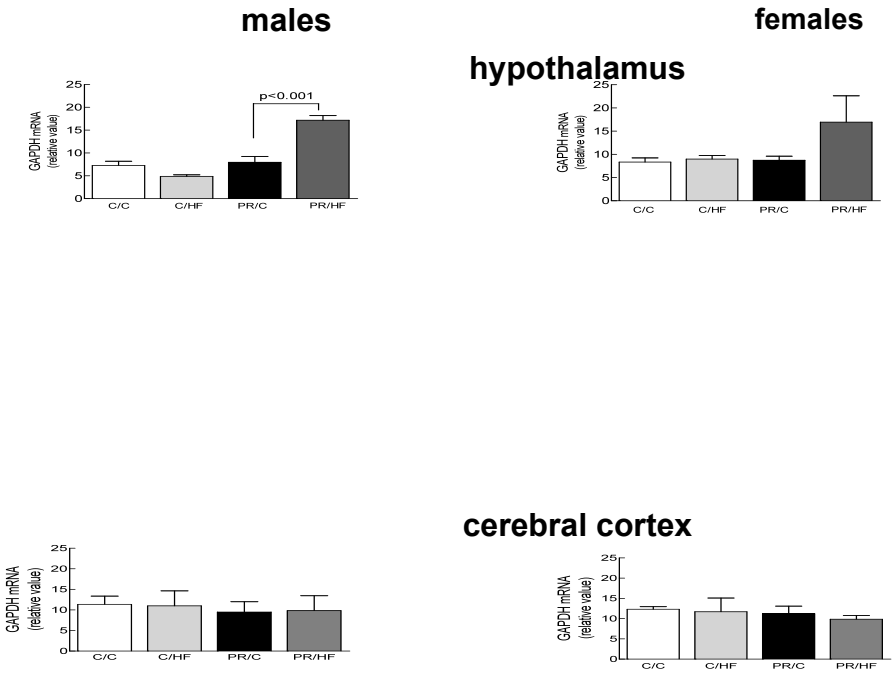


Figure 42. GAPDH mRNA expression in the hypothalamus and cerebral cortex in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group).

Hypothalamic 18s rRNA (Figure 43) levels were similar in both sexes irrespective of their prenatal and/or post weaning nutrition.

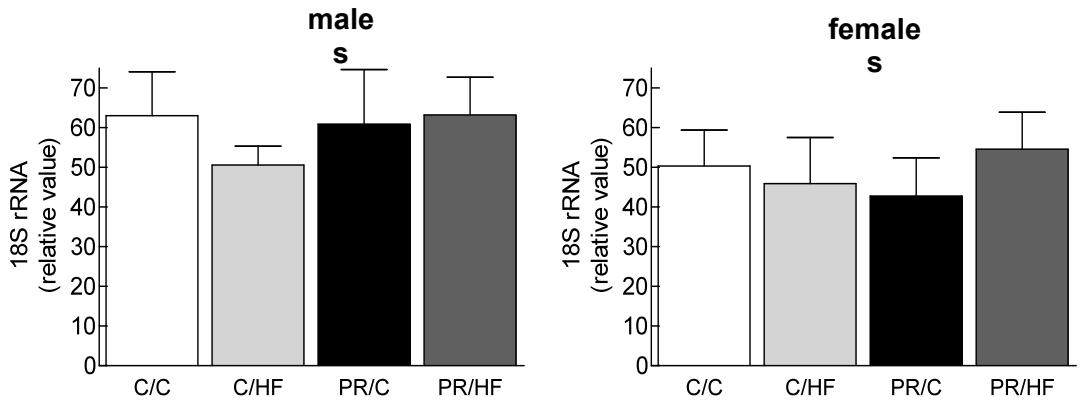


Figure 43. 18S rRNA expression in the hypothalamus in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group)

On the other hand hypothalamic β-actin (Figure 44) levels were similar in both sexes irrespective of maternal or offspring nutrition. In the cerebral cortex β-actin was also found to be stable across the experimental groups and in both sexes. β-actin is therefore a more reliable control to normalise the expression level for the appetite genes and was used as an internal standard.

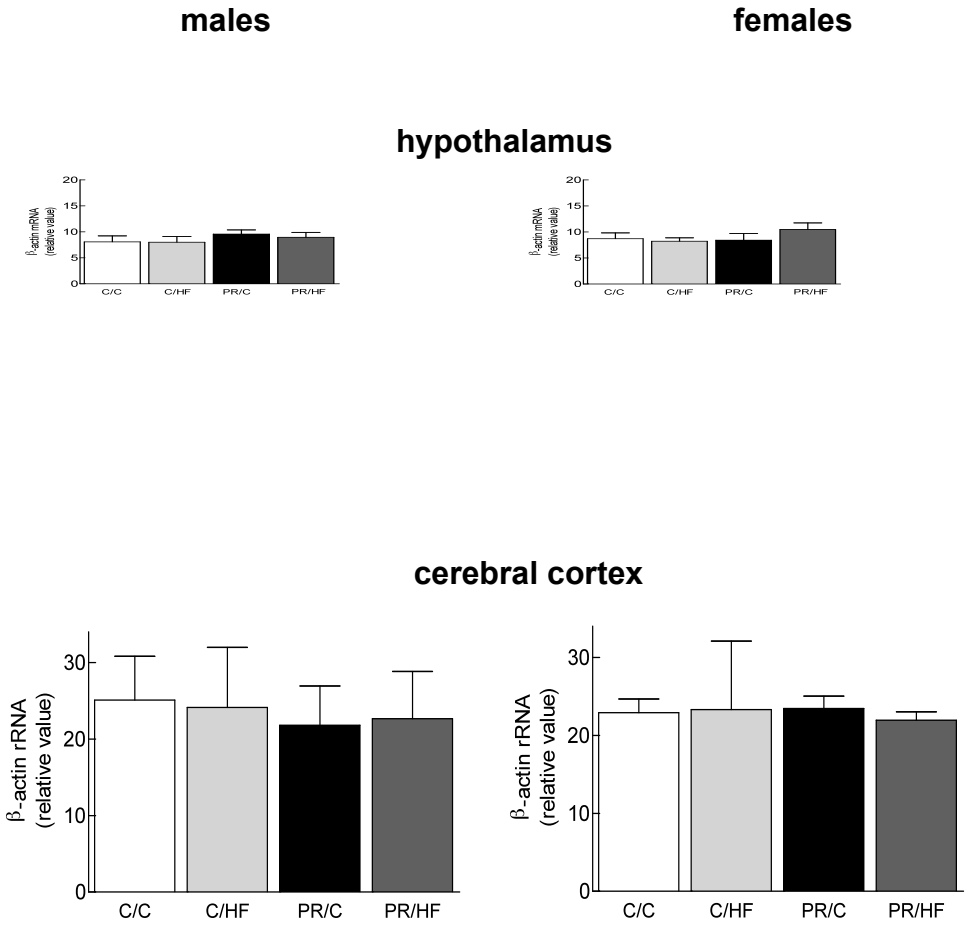


Figure 44. GAPDH mRNA expression in the hypothalamus and cerebral cortex in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group)

4.4.8.2. Hypothalamic gene expression for POMC, Ob-Rb and NPY

Marked changes in Ob-Rb and NPY mRNA expression levels were evident from the results (Figure 46a &b). In the male cohort, Ob-Rb levels were reduced by more than 50% ($p<0.05$) in the PR/HF male offspring compared to PR/C offspring. No significant changes were detected in the female offspring and no significant differences in Ob-Rb mRNA levels were found between the C/C and PR/C groups in either sex. No other significant alterations in Ob-Rb mRNA levels were seen in either the male or female experimental groups.

Significant differences in mRNA NPY expression were found (Figure 46c &d), with both the PR/HF and C/HF male groups exhibiting a two- and three-fold reduction in gene expression ($p<0.001$). NPY expression was lowered to a greater extent in the PR/HF male group than the C/HF male group. Female NPY mRNA expression levels were not significantly different between the PR/HF and C/HF groups, suggesting that NPY mRNA levels in males are more susceptible to change by a developmental nutritional insult than in females. There were no significant differences in mRNA levels of NPY between the C/C and PR/C groups (male and female).

No significant differences between experimental groups, in either the male or female cohorts were observed for hypothalamic POMC expression in my study (Figure 45e & f). In the PR/HF female group there was a trend towards an elevated expression level in the hypothalamus.

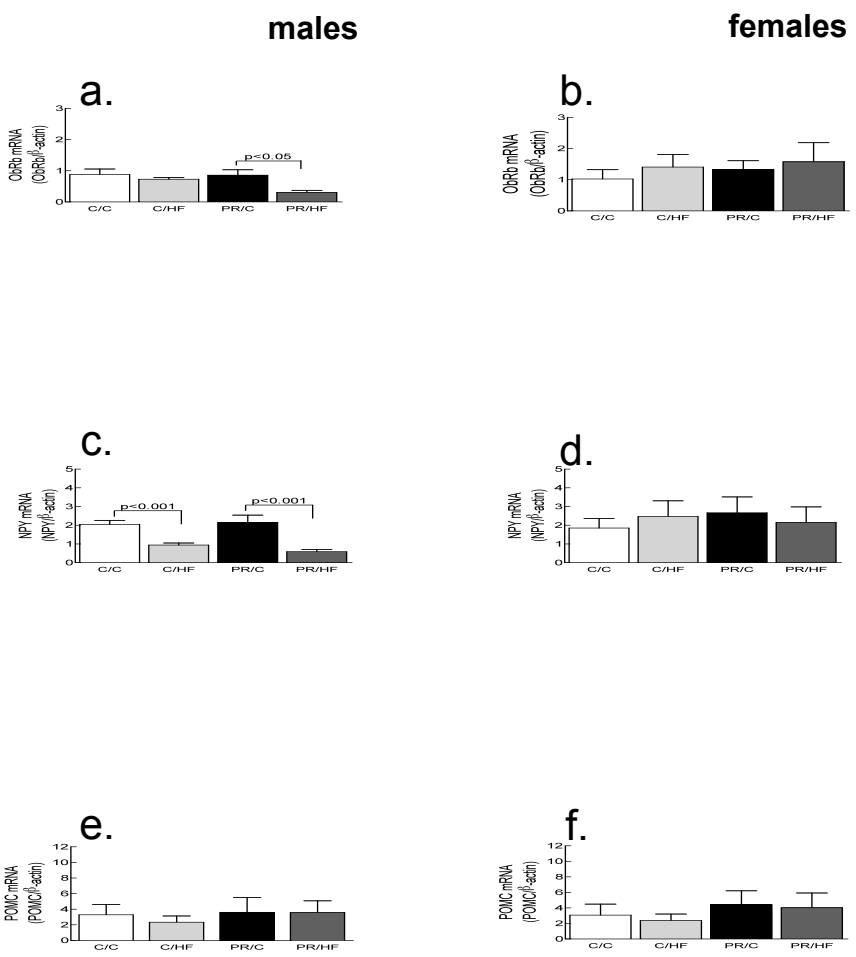


Figure 45. Hypothalamic mRNA expression levels for the leptin receptor ObRb (a & b), neuropeptide Y (NPY; c & d) and pro-opiomelanocortin (POMC; e & f) in male (a, c, e) and female (b, d, e) offspring from dams on normal protein (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M; n=7~11 per group.

4.4.8.3. UCP-1 and β-3 adrenergic receptor gene expressions in the iBAT

UCP1-1 adrenergic receptor mRNA was 60% lower in the PR/HF male and female groups ($p<0.05$) in the intrascapular brown tissue or iBAT compared to all other experimental groups (Figure 46). The other groups, namely the C/HF and PR/C offspring, were not significantly different from the C/C group in both sexes. There was a trend towards an increase in UCP-1 mRNA expression in the C/HF male and female groups but they were not found to be significant.

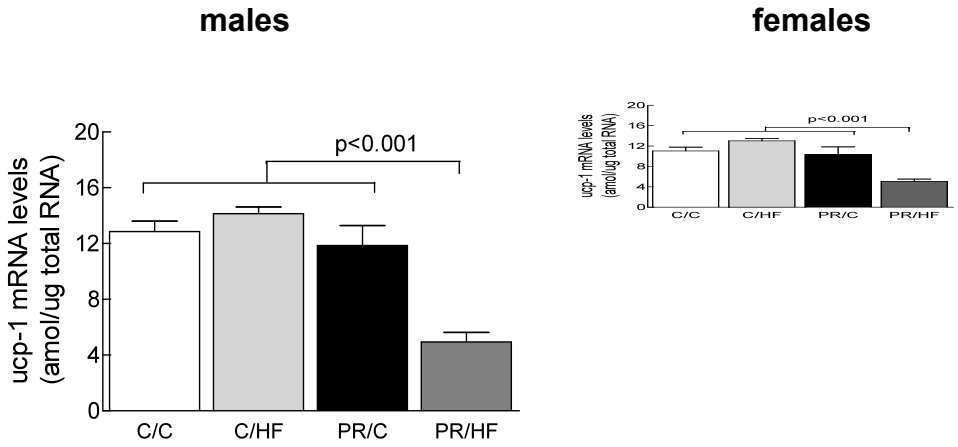


Figure 46. UCP-1 mRNA expression in iBAT in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group)

The PR/HF male group showed a 50% reduction in β-3 adrenergic receptor mRNA expression in the iBAT ($p<0.01$) compared with the PR/C males (Figure 47). No other significant differences were observed between groups. However, there was a trend towards a reduction in β-3 adrenergic receptor mRNA expression in both the PR female groups.

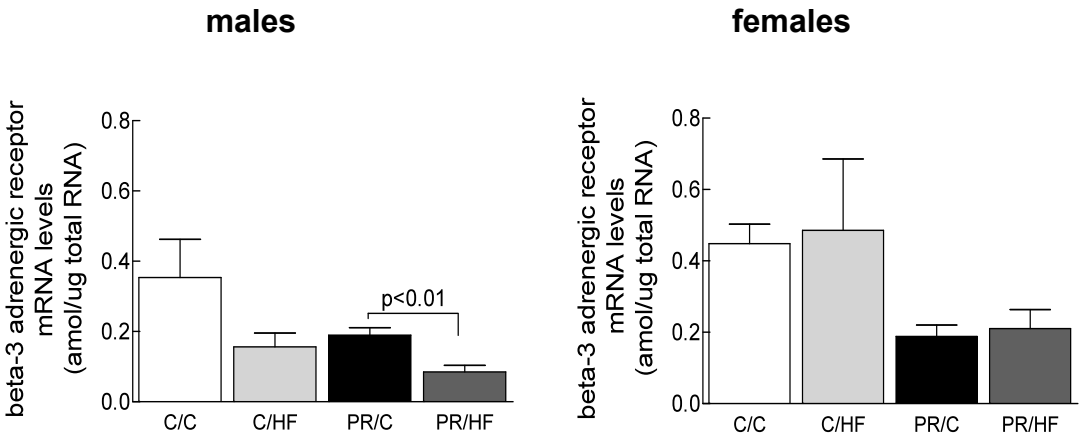


Figure 47. β-3 adrenergic receptor mRNA expression in iBAT in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=5-6 per group).

4.4.9. Correlation analysis

4.4.9.1. Total body fat and plasma leptin levels

There was a strong positive correlation between body fat and leptin levels ($p<0.0001$) in both male and female offspring (Figure 48). As total body fat increases there is a corresponding increase in plasma leptin levels.

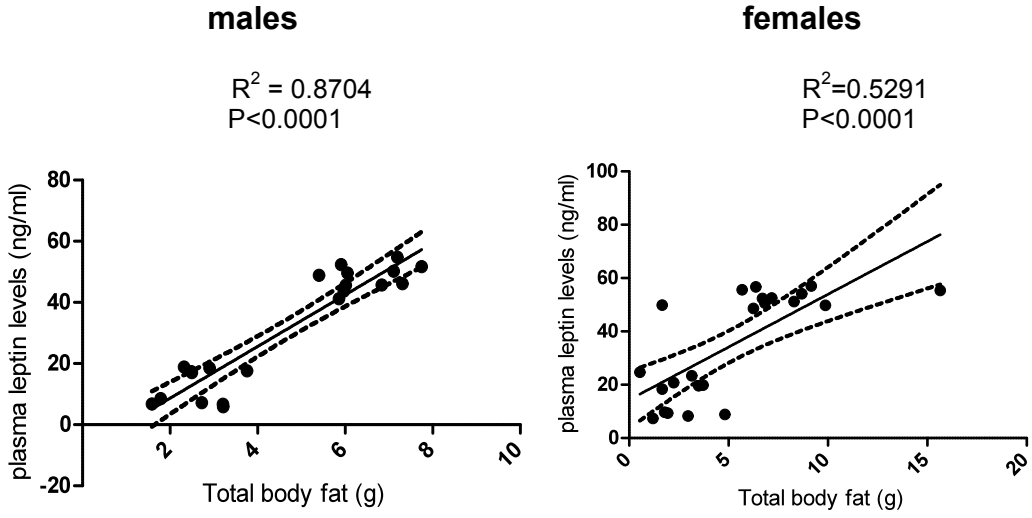


Figure 48. Correlation of total body far and plasma leptin levels in male and female offspring.

4.4.9.2. Food intake and NPY mRNA expression levels

There was a strong positive correlation ($p<0.0001$) between food intake and hypothalamic NPY mRNA expression levels in male, but not in the female offspring (Figure 50). As food intake increases, there is a corresponding increase in NPY mRNA expression in male offspring.

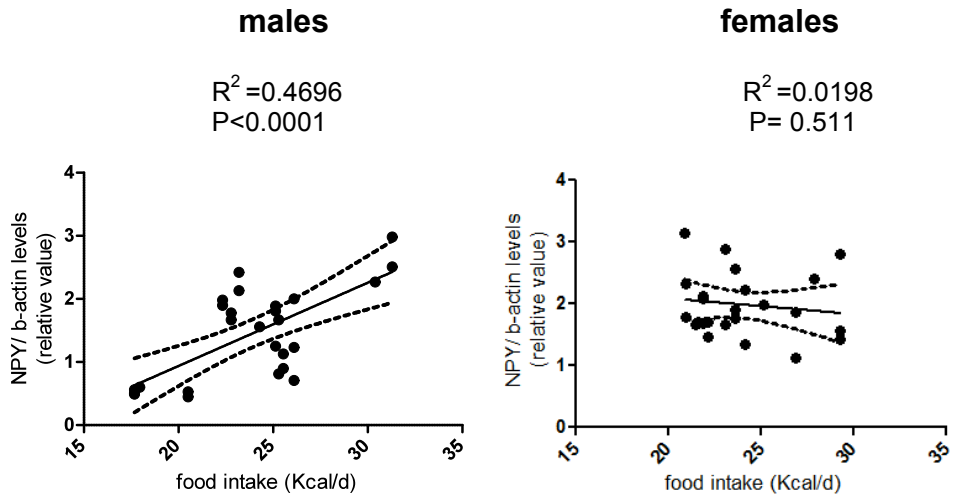


Figure 49. Correlation of food-intake and NPY levels in male offspring.

4.4.10. Maternal-offspring dietary interactions

There was a significant effect of maternal protein-restriction during pregnancy on systolic blood pressure in male ($p<0.0001$) and female ($p<0.01$) offspring (see Table 15). There was also an effect of the post weaning HF diet (offspring diet) in male ($p<0.0001$) and female offspring ($p<0.001$) on systolic blood pressure. Moreover, there was a significant prenatal-post weaning diet interaction in systolic blood pressure in both male and female offspring ($p<0.001$). No maternal diet or maternal x offspring dietary interaction effects on blood glucose and total body fat were found in male or female offspring. There was post weaning HF offspring diet effect in males ($p<0.01$) but not females on blood glucose. An offspring HF diet effects on total body fat were also found in both male and female offspring ($p<0.01$).

Table 15. Results from 2-way ANOVA analyzing maternal x offspring dietary interactions for blood glucose (BG), total body fat (TBF) and systolic blood pressure (SBP).

	Physiological parameters		
	BG	**TBF**	**SBP**
Maternal diet effect			
males	ns	ns	$p<0.0001$
females	ns	ns	$p<0.01$
Offspring diet effect			
Males	$p<0.01$	$p<0.01$	$p<0.0001$
Females	ns	$p<0.01$	$p<0.001$
Maternal x offspring diet effect			
males	ns	ns	$p<0.001$
females	ns	ns	$p<0.001$

ns = no significant difference

There was a significant maternal diet ($p<0.0001$) and maternal x offspring diet interaction ($p<0.001$) effects in both male and female offspring on UCP-1 mRNA expression in iBAT (see Table 16). There was also an offspring HF diet effect on UCP-1 mRNA expression in male ($p<0.01$) but not female offspring. There was maternal diet effect in female offspring and an offspring diet effect in males ($p<0.05$) for β-3 adrenergic receptor mRNA expression. However, I did not find a maternal x offspring diet interaction for this receptor.

Table 16. Results from 2-way ANOVA analyzing maternal x offspring dietary interactions in UCP-1 and β-3 adrenergic receptor levels.

	genes measured	
	UCP-1	**β-3 AR**
Maternal diet effect		
males	p<0.0001	ns
females	p<0.0001	p<0.05
Offspring diet effect		
males	p<0.01	p<0.05
females	ns	ns
Maternal x offspring dietary interactions		
males	p<0.001	ns
females	p<0.001	ns

ns = no significant difference

4.5. Discussion

The results in this chapter have shown that a dietary mismatch, consisting of maternal protein restriction during gestation followed by a post weaning high fat diet resulted in sex-specific reduction of food intake. Despite this observed hypophagia in the male offspring, their body weight trajectory was similar to the HF-fed males that were not exposed *in utero* to maternal dietary protein restriction. This suggest that there is an active attempt to control the rate of excess body weight gain by significantly reducing food intake in the PR/HF males, and this may be coupled with reduced energy utilization through reduced thermogenesis.

The present results suggest that the type of maternal dietary manipulation during pregnancy can result in either a hypophagic or hyperphagic phenotype in the offspring when fed post weaning a HF diet. It has been reported that severe undernutrition, such as a 70% reduction in normal food intake, to pregnant rat dams followed by post weaning HF feeding results in hyperphagia in the male offspring [26]. Hyperphagia has also been observed in rat offspring from dams who received a 50% food-restriction in utero and were normally fed post-weaning [220]. However, the results in Chapter 3 show that the food intake of offspring from pregnant mouse dams subjected to 50% global undernutrition followed by post weaning HF feeding was not altered compared with the HF-fed offspring from C-fed dams. In the present Chapter however, the HF-fed male offspring from dams that were protein restricted during pregnancy became hypophagic. These results suggest that postnatal feeding behaviour is dependent upon the extent or type of maternal undernutrition during pregnancy. Evidence suggests that serotonin activity may play a role in this differential response to maternal undernutrition. Studies in rodents have shown that maternal calorie restriction during pregnancy resulted in altered hippocampal cyanopindolol to serotonin 5-HT1B receptors, thus interfering with serotonin's inhibitory effects on appetite [221]. On the other hand, maternal protein-restriction during pregnancy

did not affect hippocampal binding activity at the 5-HT1B receptor [222]. It is still unclear however, the precise mechanisms underlying diet-induced alterations in serotonin activity. Therefore, the type of dietary insult imposed on the pregnant dams could determine the response of the offspring to post weaning HF feeding. This modification in food intake could be attributed to changes in the hypothalamic expression levels of genes involved in regulating energy intake, namely NPY and Ob-Rb. This was substantiated by the findings that hypothalamic NPY mRNA expression was strongly correlated with food intake in male offspring.

In this chapter alterations in the expression levels of the genes Ob-Rb and NPY in the hypothalamus were found in the PR/HF male offspring. It is possible that the lower NPY mRNA levels in these male offspring could be a consequence of the reduction in the numbers of NPY-expressing neurons within the hypothalamus, as has been reported in rat offspring from dams on a protein-restricted diet during pregnancy [223]. However, there was also a reduction in NPY mRNA levels in HF-fed males from chow-fed dams. Therefore the more likely explanation is that it confers some form of adaptive protection from the deleterious effect of the post weaning high fat diet in this group of offspring. Results from a previous study have shown a similar reduction in NPY mRNA expression in male mice following high fat feeding [224]. Previous studies have suggested that the reduction in NPY gene expression may be a consequence of the high plasma levels of leptin brought about by increased body weight following HF feeding [225-227]. However I found similar increases in plasma leptin levels brought about by increased body weight and adiposity following HF feeding in both the male and female offspring but only found the reduction in hypothalamic NPY mRNA levels in the males. This would suggest sex differences in the response of these neuropeptides to these dietary manipulations. Plasma leptin levels were positively correlated with adiposity in both males and females, which confirms the strong influence of fat mass on circulating leptin levels.

In contrast, no significant changes in hypothalamic POMC mRNA expression were observed regardless of prenatal and post weaning dietary manipulation or the sex of the offspring. This finding is in agreement with a study in male mice wherein POMC mRNA expression was not affected by dietary HF-feeding [227] Previous studies have also suggested that leptin could stimulate POMC gene expression in the hypothalamus [228]. It is possible that long-term HF feeding may have altered POMC sensitivity to leptin by down regulating Ob-Rb expression in POMC-containing neurons. Conversely, similar increases in plasma leptin levels brought about by HF-feeding were found in both male and female offspring. This again would suggest sex differences in the response of these neuropeptides to these dietary manipulations. Other hypothalamic neuropeptides, such as melanin-concentrating hormone, galanin and orexins, which function independently of leptin and are also responsive to dietary fat [229], may be involved in mediating the response of the PR male offspring to post weaning HF-feeding. Thus future studies could investigate the expression levels of these neuropeptides in the current mouse model.

The present study also shows that there was a reduction in hypothalamic expression of Ob-Rb in male offspring fed the HF diet, which is in agreement with previous studies [224, 230]. The predicted rise in plasma leptin levels brought about by increased body weight following HF feeding, as shown in this chapter and in previous studies [230, 229] and the reduction in hypothalamic Ob-Rb expression observed in the PR/HF male offspring should result in these animals becoming resistant to the hypophagic effect of leptin. The results however show that these males are still hypophagic. One possible explanation is that the decreased Ob-Rb mRNA levels in the PR/HF males could well contribute to the decreased expression of NPY, an orexigenic peptide [232] that co-localize with Ob-Rb expressing neurons [233], and could provide the mechanism for the reduced energy intake in these HF-fed males. However a significant reduction in Ob-Rb expression was only found in the

PR/HF males suggesting that *in utero* exposure to the maternal PR diet may have resulted in sex-specific adaptive responses of the developing neural system regulating energy homeostasis, making this system more sensitive to the post weaning HF diet. This notion is substantiated by a previous study by our group which has shown that the mRNA levels for Ob-Rb are already expressed in the fetal mouse brain and could be altered in fetuses from pregnant mice on a PR diet [195].

The absence of changes in gene expression in the females may be due to the effects of the sex hormone oestrogen on the neural system that regulates appetite [234]. Previous studies have reported sexual dimorphism in the expression of oestrogen-sensitive neurons [235, 236] and this could then influence the expression of various genes within the hypothalamus. It has also been reported that there is co-localization of oestrogen and leptin receptors on the same hypothalamic neurons [237] and studies in rats have shown that males and females display different sensitivity to central leptin administration [238]. Thus, the circulating steroid milieu in these female offspring may have some protective effect on the deleterious effect of HF feeding. In the study in this chapter, the estrus phase at the time of sampling was not standardized. Previous studies have shown that NPY and Ob-Rb could be regulated by fluctuation in ovarian steroidal milieu during the oestrous cycle [239, 240]. It is therefore possible that the lack of difference in gene expression observed in females may be a consequence of variation in oestrous cycle at the time of sampling. This may also explain why there was observed correlation between hypothalamic NPY mRNA expression and food intake in both male and female offspring, but it is only in the males that there is altered food intake. It would therefore be interesting to determine whether removing the gonads in either the male or female offspring will alter the observed results in this chapter.

As well as leptin levels, it appears that blood glucose levels may impact on adiposity. All the HF-fed offspring groups were not only the fattest but also exhibited increased blood glucose levels. There was no effect of maternal diet or maternal x offspring dietary interaction on blood glucose levels, suggesting that glucose homeostasis in the offspring is not influenced by *in utero* exposure to maternal protein restriction during pregnancy or by the prenatal-post weaning dietary mismatch. These results are particularly interesting when compared with the blood glucose results obtained in offspring from dams that were protein-restricted during pregnancy and lactation in Chapter 5.

Another interesting observation in this Chapter is the effect of the nutritional paradigm on expression levels of housekeeping genes in the hypothalamus. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) a key enzyme in the glycolytic pathway is commonly used as a control for real time PCR analysis based on the assumption that its levels are fairly constant regardless of experimental status. However several studies suggest that GAPDH mRNA levels are altered under certain nutritional states [241, 242]. The results in this chapter shows that hypothalamic GAPDH mRNA levels, but not 18S RNA and β-actin, can be altered by *in utero* exposure to maternal protein restriction coupled with post weaning HF-feeding. It is therefore not suitable to use as a control housekeeping gene in my experimental paradigm. Furthermore, a study has previously demonstrated altered hypothalamic GAPDH expression even under experimental conditions that does not involve nutritional challenges [243]. Insulin has been shown to regulate GAPDH gene expression in the absence of changes in nutritional status [244], and it is therefore likely that under conditions in which blood glucose and plasma leptin levels are altered, such as those observed in my study, that GAPDH expression would be more likely to change. Another study, specifically addressing the suitability of housekeeping genes has questioned the validity of both GAPDH and β-actin for use as internal standards [245]. This particular study revealed the variability of both GAPDH and β-actin in asthmatic

airway tissue. The authors attributed β-actin variation to non-metabolic functions, particularly its actions on structural repair during the process of airway remodelling. In my study, disproportionate cellular remodelling is a less likely outcome than metabolic disturbances. This is reflected by similar levels of β-actin among the various dietary groups. Thus, β-actin and not GAPDH was subsequently uses as a control to standardise the gene expression data.

The unchanged body weight trajectory in the hypophagic PR/HF male offspring compared to C/HF offspring suggest that they have increased energy efficiency or reduced energy expenditure. However, one cannot attribute the increased energy efficiency to reduced activity in these offspring because there were no marked changes in their locomotor activity. Moreover, altered expression of genes involved in thermogenesis was found in the intrascapular brown adipose tissue (iBAT) in these offspring. There was a reduction in UCP-1 mRNA levels in the iBAT in both male and female PR/HF offspring, but only reduced β-3 adrenergic receptor expression in the females. These changes suggest attenuated thermogenesis and reduced energy utilization in these animals. In addition, the reduction in UCP-1 expression in both males and females was dependent on maternal x offspring dietary interaction. Thermogenesis in the iBAT is initiated by the catecholamine noradrenaline which activates β-3 adrenergic receptors. This leads to the subsequent downstream activation of UCP-1 [246], the mitochondrial membrane protein involved in the critical step that allows energy to be released as heat. The concomitant reduction in expression levels for β-3 adrenergic receptor and UCP-1 is due to the influence of β-3 adrenergic receptor over UCP-1 [247]. The results from this Chapter, however, show that reduction in expression levels of UCP-1 does not necessarily equate to reduced thermogenesis, hence reduced energy utilization. Although the expression of this gene in the iBAT was significantly reduced in both male and female PR/HF animals, only the male offspring showed hypophagia with no apparent differences in their body weight trajectory,

suggestive of increased energy efficiency. Oestrogen has been shown to directly alter UCP-1 levels [248]. Thus it is possible for attenuated thermogenesis to occur independent of UCP-1 expression changes. In a recent study, a reduction in free fatty acid (FFA) availability was suggested to impair thermogenesis despite an increase in UCP-1 expression [247]. Moreover, the female sex hormone oestradiol has been shown to increase the expression levels of noradrenaline biosynthetic enzymes, which in turn stimulate the production of noradrenaline [249]. Therefore, it is plausible that the PR/HF female offspring may have higher levels of circulating noradrenaline levels. This may explain why despite the reduction in UCP-1 expression in these females there were no changes in the efficiency with which they utilize energy as was observed in male offspring.

The concept of thermoregulatory feeding in mice may explain how a chronically attenuated thermogenesis could facilitate appetite suppression [250]. Thermoregulatory feeding is the stimulation of feeding by iBAT thermogenesis and is distinct from stimulation of feeding by other stimuli such as the gut hormones. It has been shown that mice begin to eat once their core body temperature has risen, due to the necessity to produce heat over time following termination of a meal, and time of initiation of the next meal decreases. [251-253]. This increase in core body temperature, suggested to be augmented by sympathetic nervous stimulation of thermogenesis [254], causes hypoglycaemia as glucose utilization is accelerated. The drop in blood sugar levels is a peripheral signal stimulating the hypothalamus to initiate feeding. In rodents with abnormal thermogenesis, thermoregulatory feeding may also be impaired, and could be a factor in a proportion of the cases of obesity [255]. The decrease in energy utilization, and hence increased metabolic efficiency, coupled with reduced appetite observed in the PR/HF male offspring could resemble a cause-and-effect relationship whereby chronically reduced thermogenesis prevents the transient falls in core body temperature that has been shown to precede the initiation of feeding. Therefore, a state of permanent appetite

suppression predominates, which in the case of the PR/HF male offspring, may be responsible for controlling the rate of excess body weight gain despite being fed a HF diet.

The changes in metabolic efficiency seen in PR/HF male offspring may serve to promote postnatal catch-up growth. Catch-up growth is commonly considered a physiological adaptation that allows mammals to return to their genetically programmed growth trajectory following a period of growth retardation. Studies in humans have shown the deleterious health consequences of metabolic disturbances underlying catch-up growth, including cardiovascular disease [256]. However, the mechanism linking altered metabolism and cardiovascular disease remains to be elucidated. Studies in rats have demonstrated that increased fat deposition is accompanied by metabolic abnormalities that facilitate the development of such conditions as dyslipidaemia and hypercholesterolemia [257]. Studies have established that postnatal hyperphagia, which facilitates fat accumulation, occurs during catch-up growth to compensate for the *in utero* malnutrition [26]. Others however, have reported catch-up growth and elevated adiposity in the absence of hyperphagia or HF consumption [258,259]. This suggests a role for reduced energy expenditure in the increased deposition of fat.

There is much debate about the existence of brown adipose tissue (BAT) in adult humans and the role it plays in thermogenesis. It was commonly thought that adult humans possess very little BAT, which is confined to small clusters of brown adipocytes within white adipose tissue. A recent study however, using PET scans has shown that there is an abundance of BAT, albeit not in the intrascapular regions, but in supraclavicular, neck regions and small amounts within the paravertebral, mediastinal, para-aortic, and suprarenal regions in adult humans [260]. Moreover, studies in humans have confirmed the central role played by the BAT in promoting adaptive thermogenesis and increased energy expenditure [176-177], similar to its function in rodents. UCP-1, which is exclusively

expressed in BAT is the only protein known to significantly contribute to proton-uncoupling in the mitochondria [261]. This is because proton uncoupling by UCP-1 is dependent on a pair of histidine residues that appear to be absent in other uncoupling proteins. A study in humans however has demonstrated that UCP-3 has thermogenic qualities, working in the same way as UCP-1 in mouse intrascapular brown adipose tissue [262]. The difference between the two uncoupling proteins however is that UCP-3 is expressed abundantly in skeletal muscle in rodents. Thus, skeletal muscle might be the major tissue responsible for thermogenesis in humans, although this remains to be confirmed.

The nutritional mismatch employed in this study aimed to replicate the scenario common to many countries, such as India, China and Brazil, which are in transition between poverty and prosperity. While this transition may be economically favourable, it may prove less beneficial to long-term health of the population. According to the predictive-adaptive response theory, the disparity between pre- and post weaning nutrition can result in cardiovascular disease in adult offspring. I found a significant increase in systolic blood pressure in offspring with the greatest dietary mismatch, i.e. the PR/HF group. There was also a significant maternal x offspring dietary interaction in offspring, suggesting the need for both maternal protein-restriction during pregnancy and a post weaning HF diet to increase systolic blood pressure in the offspring. This may be linked to the reduced heart weights found in these offspring. Nevertheless, it remains to be determined whether reduced heart weights would equate to a reduction in heart size and altered morphology.

The present results support the predictive-adaptive response hypothesis [20] which suggests that during periods of developmental plasticity the fetus makes adaptive responses *in utero* to ensure that it survives to reproduce in the predicted postnatal environment. Such developmental alterations confer advantages on the fetus only if the

prediction is accurate. However, if the actual postnatal environment diverges from what was predicted, the offspring phenotype becomes inappropriate and disease and metabolic dysfunction manifest. I have demonstrated studies done in this Chapter that a disparity between prenatal and post weaning nutrition has a profound effect on food intake and is reflected by alterations in the expression of genes that regulate appetite. In addition to affecting energy intake, the dietary mismatch appears to modify the offspring's metabolism as well. These concepts may have profound implications to the development of obesity and related metabolic conditions, particularly in developing countries undergoing rapid demographic, epidemiological and nutritional transition due to increasing economic prosperity.

In conclusion, the results in this chapter have shown that mismatched prenatal and post weaning diets lead to sex-specific dysregulation of energy homeostasis. This is characterized by changes in the expression of genes involved in regulating food intake, which in the male offspring, may provide some form of adaptive protection against an energy-rich postnatal environment. Such changes are not seen in female offspring and may result from different life-course strategies in relation to fat accretion necessary for reproductive function [263-265].

Both the current and previous chapters have examined the effects of maternal undernutrition during pregnancy coupled with post weaning HF feeding on the metabolic and cardiovascular phenotype of the offspring. Little is known about the role of this type of maternal x post weaning nutritional mismatch if the maternal undernutrition is extended to include the lactation period. The subsequent chapter therefore examines the effects of maternal protein-restriction during pregnancy and lactation followed by a post weaning HF diet, on the metabolic and cardiovascular phenotype of the offspring.

Chapter 5

Effects of maternal protein-restriction during pregnancy and lactation followed by a high-fat post-weaning diet on the metabolic and cardiovascular phenotype of offspring

5.1. Introduction

It is clear from both animal and epidemiological studies that early life provides a period of developmental plasticity that allows for adaptation to the perceived nutritional environment [14, 20, 26]. The results shown in the preceding Chapters, as well as a number of rodent models of maternal undernutrition during pregnancy, have demonstrated altered cardiovascular and metabolic phenotype in adult offspring [59]. However, it has also been shown that maternal protein restriction during the lactation period could alter metabolic and cardiovascular phenotype in their offspring [266]. The importance of imposing maternal protein restriction to both the pregnancy and lactation periods to offspring health remains to be elucidated. In rodents, the late gestation and early postnatal periods are believed to be critical in determining the cardiovascular phenotype of the offspring. This period coincides with nephrogenesis (i.e. the development and growth of the kidneys), which lasts until about two weeks after birth [267]. Hypertension resulting from maternal undernutrition during pregnancy can be reversed by adequate nutrition during lactation [268]. This clearly demonstrates the importance of the lactation period in the development of the cardiovascular system. Maternal protein-restriction extending into lactation in mice has also been shown to have deleterious effects on other organs, such as the pancreas [64]. Pancreatic β-cell proliferation continues until postnatal life [269], therefore this period is vital for normal pancreatic development. Conversely, skeletal muscle development in mammals does not continue after birth [270]. Thus, maternal undernutrition during the lactation period has no effect on skeletal muscle number and function in adult offspring Therefore when examining how the gestation or the lactation period impacts on the future health of the offspring, one should bear in mind the organ being studied and the timing of its development. If the organ's development occurs predominantly during lactation, then a nutritional insult during this period would be more consequential to organ dysfunction and subsequent disease than if the insult was only limited to the gestation period. A study done in rats [271] has shown that maternal protein

restriction during the lactation period impacts more on the offspring's post weaning growth trajectory than protein restriction imposed on the dam during the pregnancy period. It remains to be elucidated however, what organs and tissue are affected and responsible for the altered growth trajectory in these offspring. This chapter will try to identify some of the physiological mechanisms underlying the phenotypic changes observed in the offspring and also examine whether changes in their growth trajectory are accompanied by cardiovascular and metabolic dysfunction.

Body weight regulation is controlled predominantly within the hypothalamus. This region of the brain contains populations of neurons which express neuropeptides involved in appetite regulation and energy expenditure [272]. Given that neural connections in the hypothalamus are not yet fully established at the time of birth, it is not surprising that environmental factors such as maternal undernutrition during lactation could influence offspring body weight trajectory in adulthood.

This Chapter also examined the effects of programming of food preference in the offspring. A previous study showed an increased preference for high-fat food amongst offspring from dams that were protein-restricted exclusively during the pregnancy period [58]. The authors suggested impairment not only of the mechanisms controlling appetite within the hypothalamus but also that of the regions of the brain controlling palatability and smell.

The mechanisms and circuitry involving energy expenditure regulation are somewhat more complex than those involved in regulating food intake. The circuitry involved in energy expenditure regulation not only involves the hypothalamus but also includes peripheral organs such as the adipose tissue [273]. Therefore, while environmental factors imposed during the lactation period could impact on hypothalamic mechanisms that regulate energy

expenditure, one must also consider its impact on peripheral organs involved in energy expenditure regulation. Adipogenesis, the process of adipose tissue growth and development, begins in late gestation and continues after birth. The lactation period is particularly important for the development of brown adipose tissue (BAT), which has important functions related to heat production in newborn mammals [274]. Up to 80% of the BAT is laid down during the lactation period. The lactation period also coincides with the development of sympathetic nervous system innervations to the BAT [273], which in mice occurs during the lactation period [273] and is responsible for the activation of the thermogenic qualities of the BAT [275]. Given that BAT thermogenesis is central to energy expenditure regulation, the lactation period therefore is not only critical for the development of mechanisms that regulate not only food intake but also those involved in the regulation of energy expenditure. Adverse nutrition during this period is therefore likely to influence long-term energy expenditure regulation.

While others have shown that extending maternal protein restriction to include the lactation period could alter body weight trajectory of the offspring [271], it remains unclear whether post weaning overnutrition, such as feeding a high fat diet, has deleterious consequences on the offspring phenotype. In this Chapter, a HF post weaning diet was given to the offspring from dams that were protein-restricted during pregnancy and lactation.

5.2. Aims

1) To determine the metabolic and cardiovascular phenotype and food preference of adult offspring from dams that were protein restricted during pregnancy and lactation.

2) To examine whether there are changes in the expression of genes involved with appetite regulation in the hypothalamus and those involved in thermogenesis in the iBAT of adult offspring from dams that were fed a protein restricted diet during pregnancy and lactation.

3) To find out the effects of post weaning high-fat feeding on the cardiovascular and metabolic phenotypic outcomes and gene expression patterns in the adult offspring from dams that were protein-restricted during pregnancy and lactation.

4) To find out whether these changes are sex-specific.

5.3. Methods

5.3.1. Experimental procedures

All procedures were carried out in accordance with the UK Animal Scientific Procedures Act of 1986. Female MF-1 mice were individually housed under a 12h light-dark cycle and given full access to drinking water throughout the study. These females were time mated at 8-10 weeks of age, and on confirmation of pregnancy by the presence of vaginal plug, were randomly divided into two dietary groups (see Figure 51). One group (n=10) was fed a standard chow (C) diet that contained 18% casein and the other (n=10) was fed a protein-restricted (PR) diet containing 9% casein. These diets were isocaloric (see General Methodology in Chapter 2 for details of the C and PR diet). Both sets of dams were fed their respective diets throughout the gestation and lactation periods. At birth litter size was standardized to 8 pups per litter. Three weeks after birth, offspring from both sets of dams were weaned onto either the C or a high fat (HF) diet (see General Methodology in Chapter 2 for details of the HF diet).

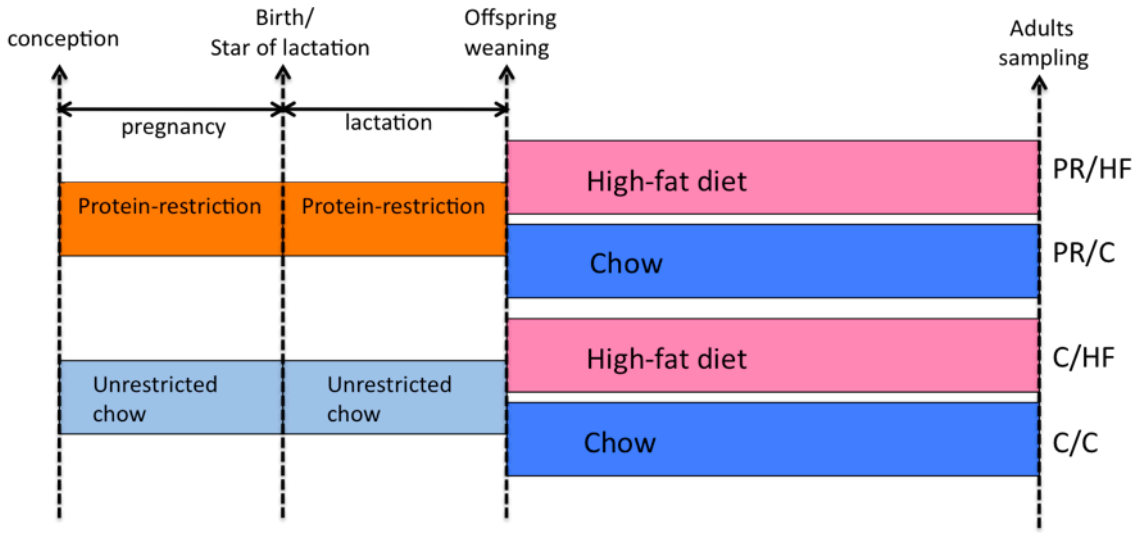


Figure 50. Experimental protocol. Pregnant dams were fed a protein restricted (PR) diet or standard chow (C) diet throughout the pregnancy and lactation period. Offspring from both sets of dams were weaned onto a high fat (HF) diet (generating dam/offspring dietary groups PR/HF and PR/C), or the C diet (generating dam/offspring dietary groups C/C and C/HF).

Food intake and body weight of the offspring were monitored during the course of the study. Between 28-30 weeks of age the offspring's metabolism and blood pressure were measured by indirect calorimetry and tail-cuff plethysmography, respectively (see General Methodology in Chapter 2 for details). Adult offspring were humanely killed at 32 weeks of age by CO_2 inhalation and cervical dislocation. Each offspring group had a sample size of between 7-11 animals. Blood was collected by cardiac puncture and fat depots (i.e. gonadal, retroperitoneal, interscapular, inguinal and peri-renal) were dissected and weighed. Cumulative fat depot weights for each animal were compared to their total body weights and body fat, as a percentage of total body weight, was calculated

5.3.2. Food preference study

Animals at 30 weeks old were singly housed and given free access to three different food sources. This was to determine whether their preference for food high in protein, fat or carbohydrates has been altered by *in utero* and lactational exposure to maternal protein restriction. The different diets were colour coded to allow for accurate measurement of food intake and were provided as balls or pellets and placed in the cage at random positions each day. The mice were allowed to become accustomed to the self-selection regimen for two days before food intake was measured. Food intake was monitored twice daily for the duration of the diet self-selection period. A previous study in rats has reported that a period of 72 hours was sufficient to obtain an accurate account daily estimated food intake in adult rodents [58]. The present study however conducted the self-selection food trial for 5 days to give a more accurate assessment of daily food intake. The nutrient composition of the self-selection diets are shown in Table 17.

Table 17. Nutrient composition of the high-protein, high carbohydrate and high-fat diets used in the self-selection food trial.

	High fat diet (weight per 100g)	High carbohydrate diet (weight per 100g)	High protein diet (weight per 100g)
Protein	20g	18g	40g
Fat	45g	20g	20g
Carbohydrate	35g	62g	40g

5.3.3. Gene expression analysis

5.3.3.1. Hypothalamic gene expression analysis for NPY, POMC and Ob-Rb

Total RNA was extracted from hypothalamic tissue samples and cDNA was synthesized. Specific primers and probe for NPY, POMC, Ob-Rb, β-actin were designed based on their published sequences (from PUBMED) using the primer express™ (v1.0) software. Oligonucleotide sequences were synthesized by Eurogentec Ltd (Romsey UK). The primer and probe oligonucleotide sequences for β-actin, NPY, POMC and Ob-Rb in Tables 7, 9, 10 & 11, respectively, in Chapter 4. Gene transcript levels were then measured by RT-PCR (see General Methodology in Chapter 2 for details of protocol). β-actin was used to normalize expression levels of mRNA for NPY, POMC, and OB-Rb

5.3.3.2. Gene expression analysis for UCP-1 and β-3 adrenergic receptor in the interscapulary brown adipose tissue (iBAT)

Gene expression analysis for UCP-1 and β-3 adrenergic receptor in the iBAT was measured by RT-PCR (see General Methodology in Chapter 2 for details of protocol). The primer sequences for UCP1 and β-3 adrenergic receptor were designed using the Universal Probe Library™ website (Roche: https://www.roche-applied-science.com/sis/rtpUN/upl/index.jsp). The primer sequences for UCP-1 and β-3 adrenergic receptor are shown in Tables 18 and 19, respectively.

Table 18. β-3 adrenergic receptor primers & probe sequences

Forward (sense) Primer	5'- CAG CCA GCC CTG TTG AAG-3'
Reverse (anti-sense) Primer	5'- CCT TCA TAG CCA TCA AAC CTG-3'
Probe	Universal probe #13, cat.no. 0468512100

Table 19. UCP-1 primers & probe sequences

Forward (sense) Primer	5'- GGC CTC TAC GAC TCA GTC CA-3'
Reverse (anti-sense) Primer	5'- TAA GCC GGC TGA GAT CTT GT-3'
Probe	Universal probe #13, cat.no. 0468512100

5.3.4. Histological analysis of intramuscular fat deposition

Skeletal muscle (gastrocnemius) was dissected from the lower back limb of the animal and snap frozen in liquid nitrogen and stored at -80ºC for later use. Whole muscle was set in Tissue-Tek® O.C.T. (Optimal Cutting Temperature) compound (Sigma Chemicals Ltd UK) and transversely cut into 7 micron thick sections using a cryostat. Sections were thaw-mounted onto super-frost slides. Oil Red O (Sigma Chemicals Ltd UK) was used to stain the muscle sections to detect intramuscular fat deposition. Gill's haematoxylin (Sigma Chemicals Ltd UK) was used as a counterstain to aid in the visualisation of Oil Red O staining within the muscle sections. Sections were viewed under the microscope. The KS300 image analysis software (Zeiss, Germany) was used to determine adipocyte sizes and numbers as well as intramuscular lipid content and muscle fibre and nuclei numbers per muscle cross-sectional area. Adipocyte sizes and numbers were measured in five different microscopic frames randomly chosen such that between 160 and 400 sections were measured from each sample. Intramuscular fat area was measured in the whole semitendinosus cross-sectional area. Muscle fibres and nuclei were counted in five microscopic frames per section chosen randomly and represented 7–10% of the whole muscle cross-sectional area. The KS300 software was used to calculate the cumulative area of each section examined which was stained red (i.e. adipose tissue).

5.3.5. Statistical analysis

All values are presented as mean ± SEM. Effect size estimates for body weight gain and energy intake are from a mixed model analysis [161] that considers all time points through the study, controlling for the set of dam-pup relationships. All other data were analyzed statistically using analysis of variance (ANOVA) followed by the Tukey-Kramer test for comparisons where appropriate. Statistical significance was assumed if $P<0.05$. Certain phenotypic parameters (i.e. total body fat, energy expenditure, systolic blood pressure and intramuscular fat) were analyzed by two-way ANOVA for maternal x offspring diet interaction. Mixed-model analysis and two-way ANOVA was done with SPSS 14.0 (SPSS Inc). All other analysis was done using Sigma Stat (Systat Software Inc) or Prism (GraphPad Software Inc) statistical programs.

5.4. Results

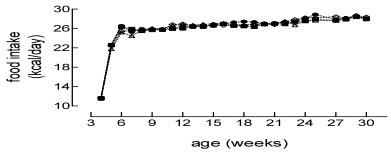
5.4.1. Food Intake & body weight

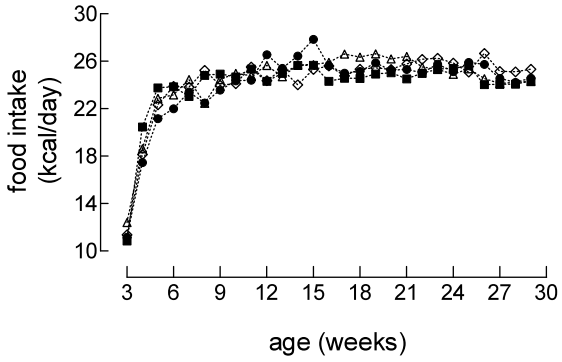
In males, the PR/HF group exhibited significant reduction in body weight trajectory compared to the C/HF group ($p<0.001$, see Figure 51A and Table 20). This difference was more apparent from about 9 weeks of age. On the other hand, the body weight trajectory of the PR/HF males was greater than the PR/C group ($p<0.001$), which in turn was similar to the C/C male offspring group. In females, the PR/HF animals also had lower body weight trajectory compared to C/HF group ($p<0.001$), but were significantly greater compared with the PR/C animals ($p<0.001$). Unlike in the male offspring however, the PR/C females had greater body weight trajectory than the C/C group ($p<0.001$). This was more apparent from about 24 weeks of age.

males **females**

A.

B.





Maternal diet/offspring diet: ■ C/C △ C/HF ◇ PR/C ● PR/HF

Figure 51. (A) Body weight gain and (B) food intake (kcal/day) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

Table 20. Estimate of mean difference and 95%CI of body weight and energy intake in the offspring

Group comparisons		Variables			
		body weight, g		energy intake, kcal/day	
		males	females	males	females
PR/HF vs PR/C	Mean	9.08	2.61	0.3	0.91
	95% CI	2.64, 11.85	0.55, 3.47	-0.68, 1.22	-0.45, 1.18
	P value	<0.001	<0.001	ns	ns
PR/HF vs C/HF	Mean	8.38	2.77	0.25	0.02
	95% CI	5.636, 11.14	1.1, 2.94	0.01, 1.14	-0.19, 0.84
	P value	<0.001	<0.001	ns	ns
PR/C vs C/C	Mean	0.215	1.06	0.01	0.15
	95% CI	-2.5, 2.93	-0.82, 1.84	-0.66, 0.4	-1.17, 1.34
	P value	ns	<0.001	ns	ns

Effect estimates (n = 7-11 per group) are from a mixed model analysis that considers all time points through the study, controlling for the set of dam-pup relationships. ns = no significant difference.

5.4.2. Food preference of offspring from dams following maternal protein restriction during pregnancy and lactation

Maternal protein-restriction during pregnancy and lactation had no significant effect on offspring preference for a particular type of diet in both males and females (Figure 52). Regardless of sex and maternal diet during pregnancy and lactation, all offspring groups demonstrated a 5 fold preference for fat preference for a fat-rich diet compared to either one high in protein or high in carbohydrates ($p<0.05$).

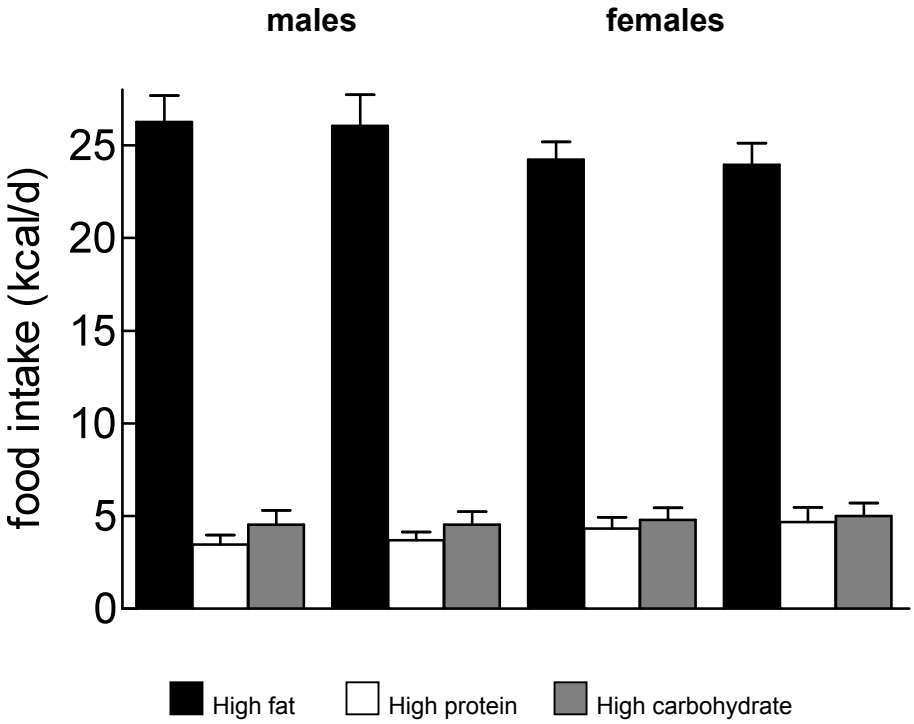


Figure 52. Selection of nutrient resources by male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C. All values are expressed as means ± S.E.M (n=7-11 per group)

5.4.3. Adiposity

5.4.3.1 Total body fat (% total body weight)

In male HF-fed offspring from dams that were fed a C diet during pregnancy and lactation (C/HF), there was an increase in total body fat compared with the C/C group (see Figure 53, $p<0.001$). Male PR/HF offspring exhibited a greater amount of total body fat than both PR/C and C/HF male offspring groups ($p<0.001$). Interestingly, there was no difference in total body fat between the C/HF and the PR/C males. In the female cohort, C/HF offspring were fatter than the C/C offspring ($p<0.001$). Although there seems to be reduced adiposity in the PR/C and PR/HF groups compared with the C/HF females, this was not found to be significantly different.

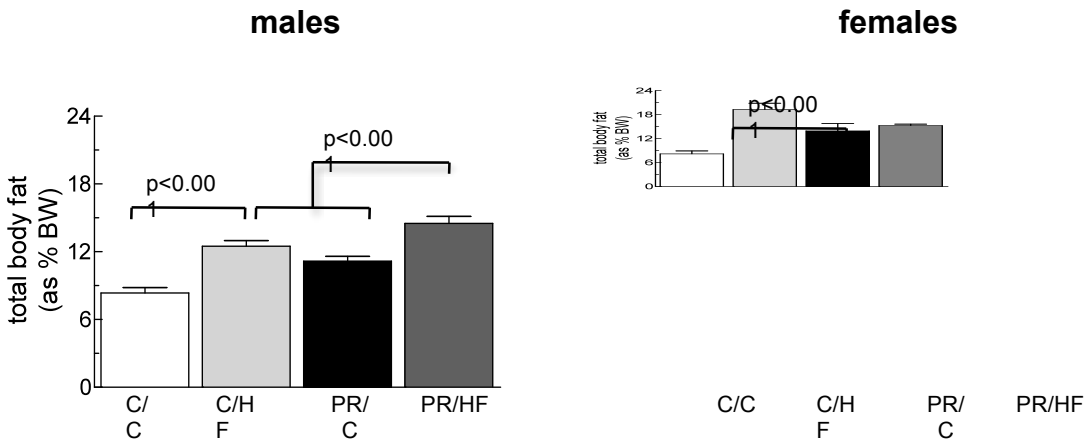


Figure 53. Total body fat (expressed as % of total body weight) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.3.2 Individual fat depots

Male PR/HF offspring exhibited greater amounts of gonadal and inguinal fat compared to C/HF offspring ($p<0.05$, see Figure 54). PR/C male offspring exhibited greater amounts of gonadal and retroperitoneal fat compared to C/C male offspring ($p<0.05$) (see Figure 55). Female PR/HF offspring have greater amounts of gonadal and inguinal fat compared to PR/C offspring.

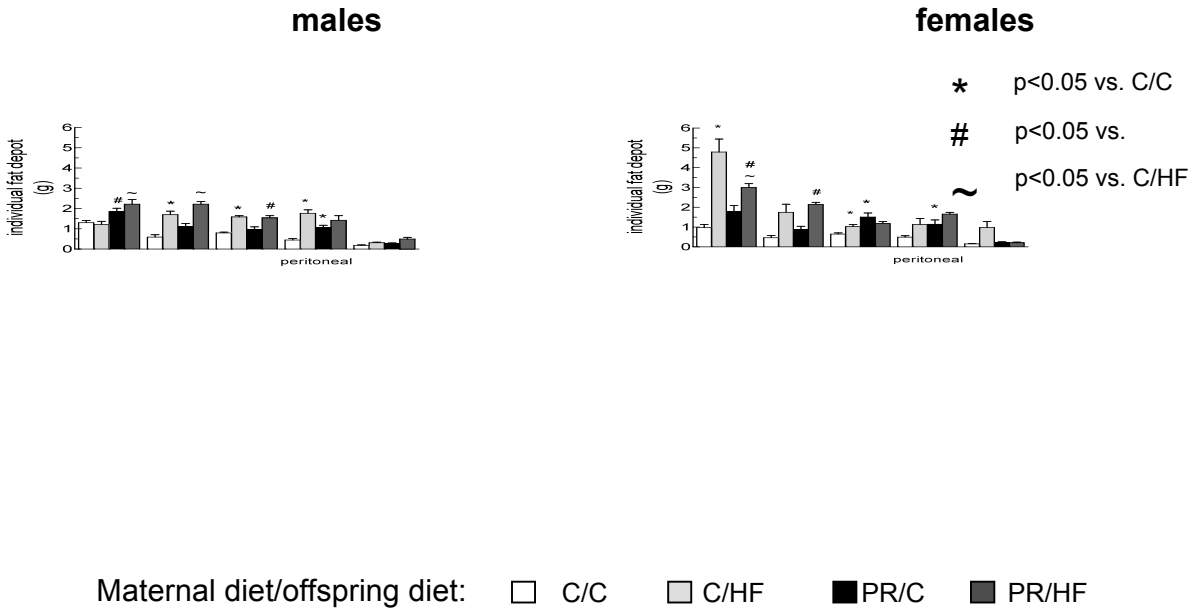


Figure 54. Weight (g) of individual fat depots in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.4. Intramuscular fat deposition

There was a significant increase in intramuscular fat in the C/HF male offspring compared with the C/C animals ($p<0.01$, see Figures 55 and 56). On the other hand, as with the C/C offspring, there were no increases in intramuscular fat deposition in the PR/C group. Nevertheless, the PR/HF offspring showed the greatest amount of intramuscular fat deposits, which was more than what was observed in the C/HF animals.

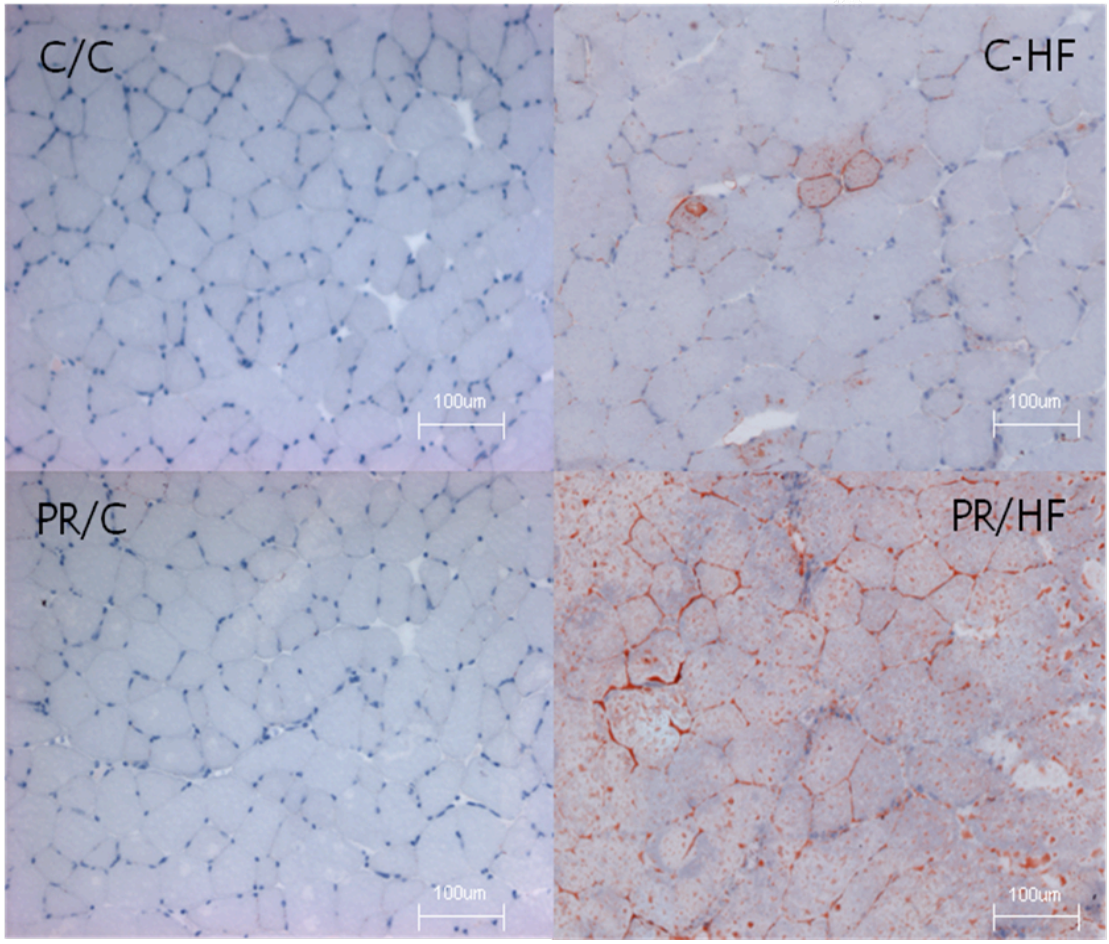


Figure 55. Representative images of intramuscular fat deposition established by Oil Red O staining of the skeletal muscle in male offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF.

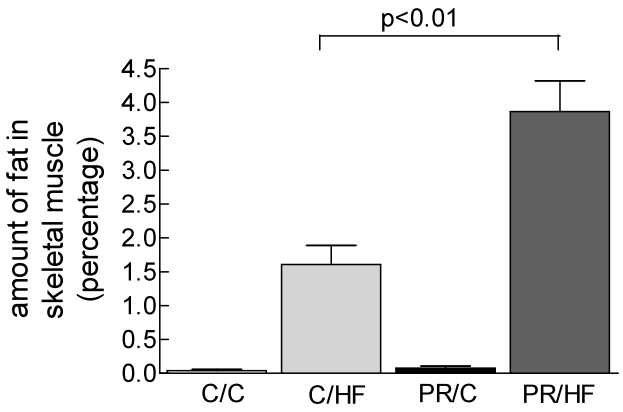


Figure 56. Amount of fat in skeletal muscle in male offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.5. Systolic blood pressure

Systolic blood pressure was elevated by 15% in the C/HF male and female offspring compared to C/C groups ($p<0.05$, see Figure 57). On the other hand, PR/C male and female offspring had higher systolic blood pressure compared with C/C and C/HF groups, respectively ($p<0.05$). In the PR/HF male and female offspring, systolic blood pressure was further elevated by more than 30% and 15% compared to male and female PR/C offspring, respectively ($p<0.05$).

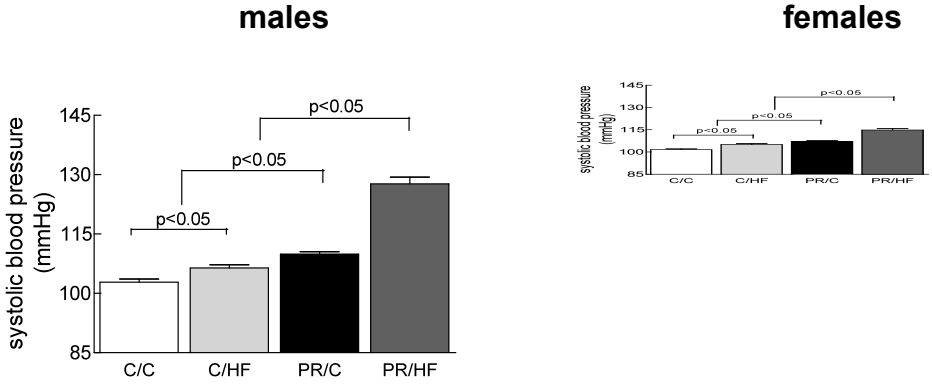


Figure 57. Systolic blood pressure in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.6. Energy expenditure and Respiration Quotient (RQ) values

In males but not females, PR/HF offspring exhibited reduced energy expenditure (see Figure 58) compared with other treatment groups ($p<0.05$). No significant differences were observed between offspring groups in the female cohort.

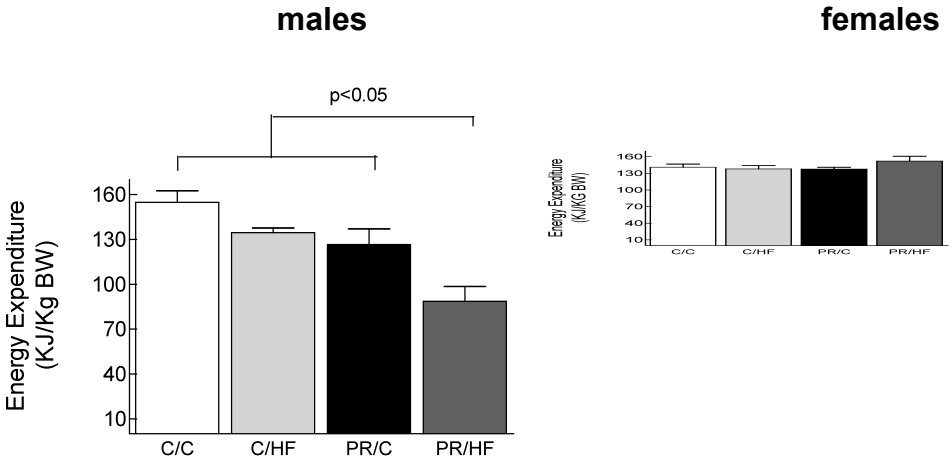


Figure 58. Energy expenditure (Kj/KG) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

In both male and female offspring, there were reduction in respiration quotient (RQ) values following HF feeding irrespective of the dam's diet during pregnancy and lactation ($p<0.001$, Figure 59). Moreover there were no differences in RQ values between offspring from the C-fed and PR dams.

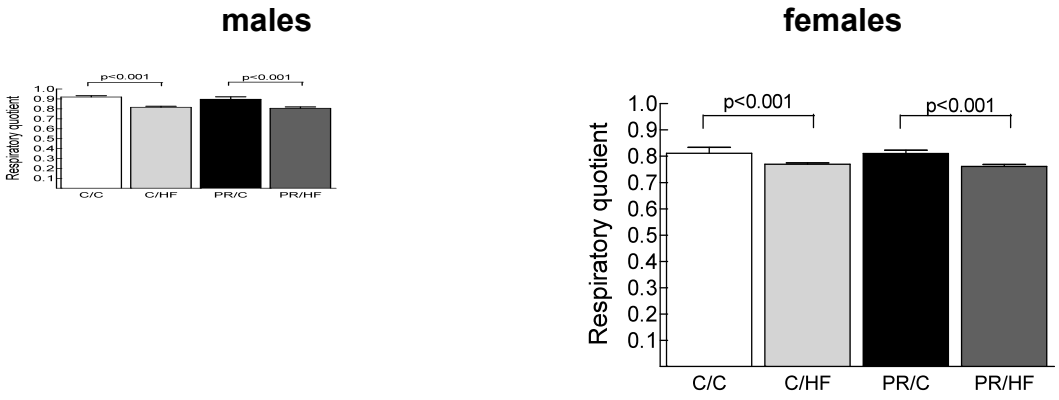


Figure 59. Respiration quotient (RQ) values in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.7. Blood glucose levels

In male offspring, PR/HF males exhibited increased blood glucose levels compared with PR/C and C/C offspring ($p<0.05$, see Figure 60). Female PR/HF, PR/C and C/HF offspring exhibited increased blood glucose levels compared with C/C offspring ($p<0.05$).

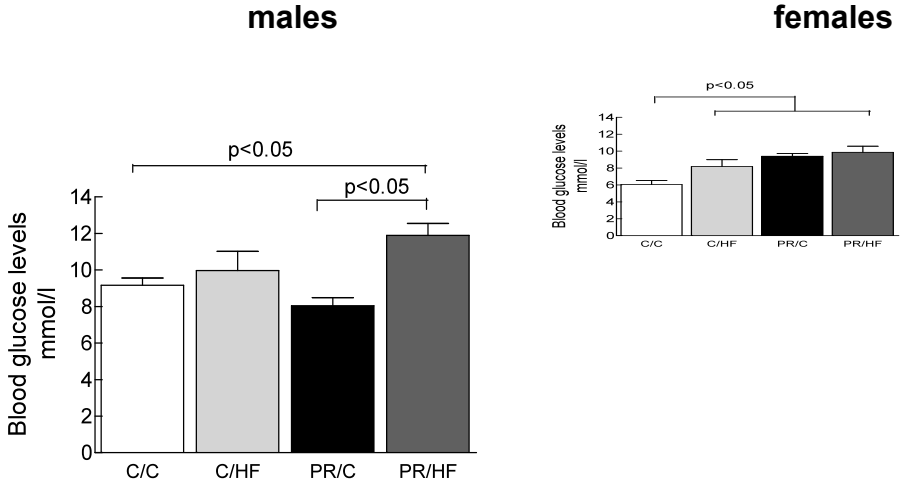


Figure 60. Blood glucose levels (mmol/l) in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.8. Gene analysis

5.4.8.1. Gene expression in the hypothalamus

No significant differences in mRNA expression of NPY and POMC were observed between experimental groups in both male and female offspring group (Figure 61).

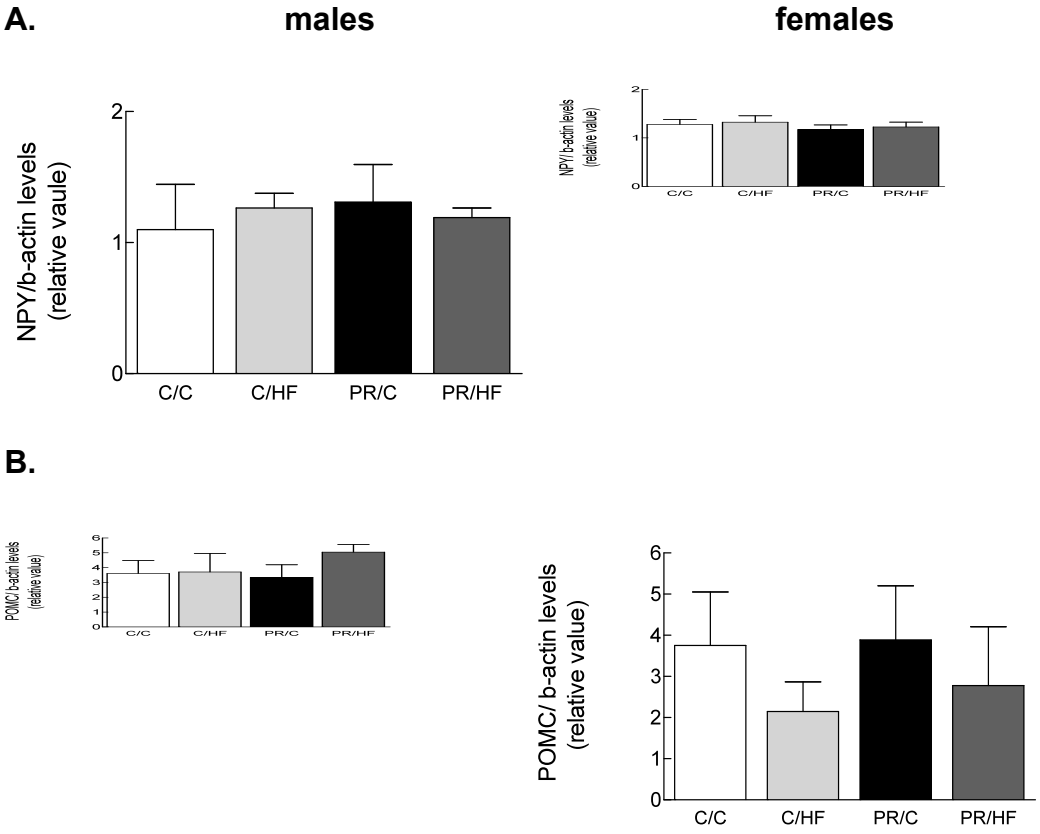


Figure 61. (A) NPY and (B) POMC mRNA expression in the hypothalamus in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11 per group).

5.4.8.2. Gene expression in the iBAT

In males, iBAT UCP-1 mRNA levels were 75% higher in C/HF males compared with C/C males ($p<0.05$, see Figure 62). The iBAT UCP-1 levels were also lower in the PR/C and PR/HF male offspring groups compared with the C/HF males ($p<0.05$). In females, there were no significant changes in UCP-1 mRNA levels in the iBAT among the 4 experimental groups.

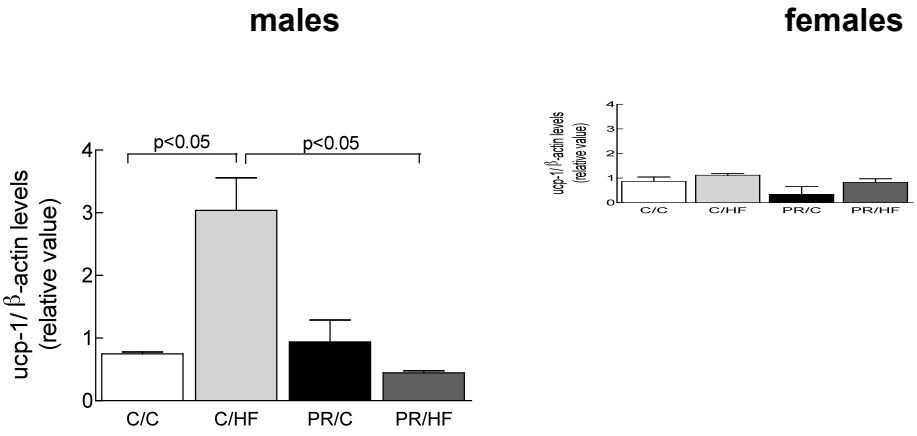


Figure 62. UCP-1 mRNA expressions in the iBAT in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11per group).

β3 adrenergic receptor (β-3 AR) mRNA expression levels were elevated in the C/HF males compared with the other experimental groups ($p<0.01$, see Figure 63). There was a further 50% reduction in β-3 AR mRNA expression in the PR/HF group, but this was not found to be significantly different compared with the C/C and PR/C animals. On the other hand, there were no significant changes in β-3 AR mRNA levels in the females for all the experimental groups.

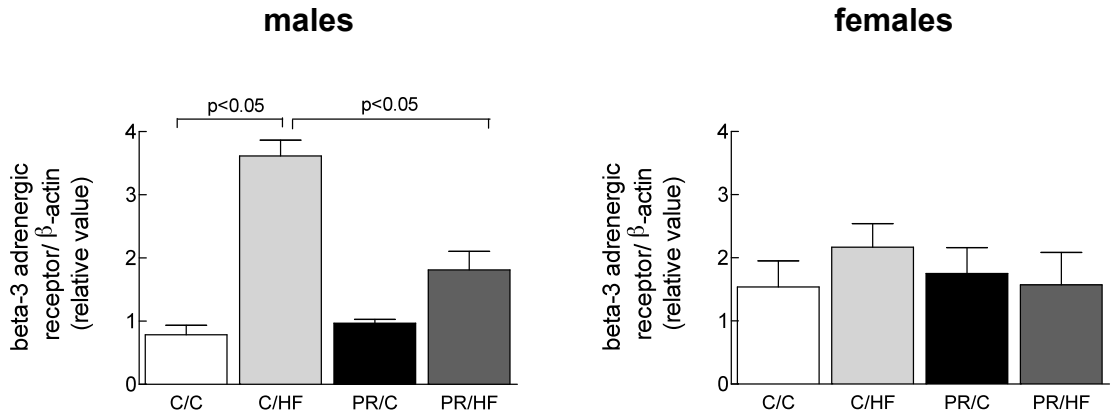


Figure 63. β-3 adrenergic receptor mRNA expression in iBAT in male and female offspring from dams on standard chow (C) or protein restricted (PR) diet during pregnancy and lactation. Weaned offspring were then fed either a high fat (HF) or C diet to adulthood, thus generating the dam/offspring dietary groups: C/C, C/HF, PR/C and PR/HF. All values are expressed as means ± S.E.M (n=7-11per group).

5.4.9. Correlation analysis

5.4.9.1. Total body fat and blood glucose levels

There was a positive correlation between blood glucose levels and total body fat in male offspring ($p<0.01$, see Figure 64). As total body fat increases, there is a corresponding increase in blood glucose levels. In the female however, there were no significant correlation between body fat and blood glucose in female offspring.

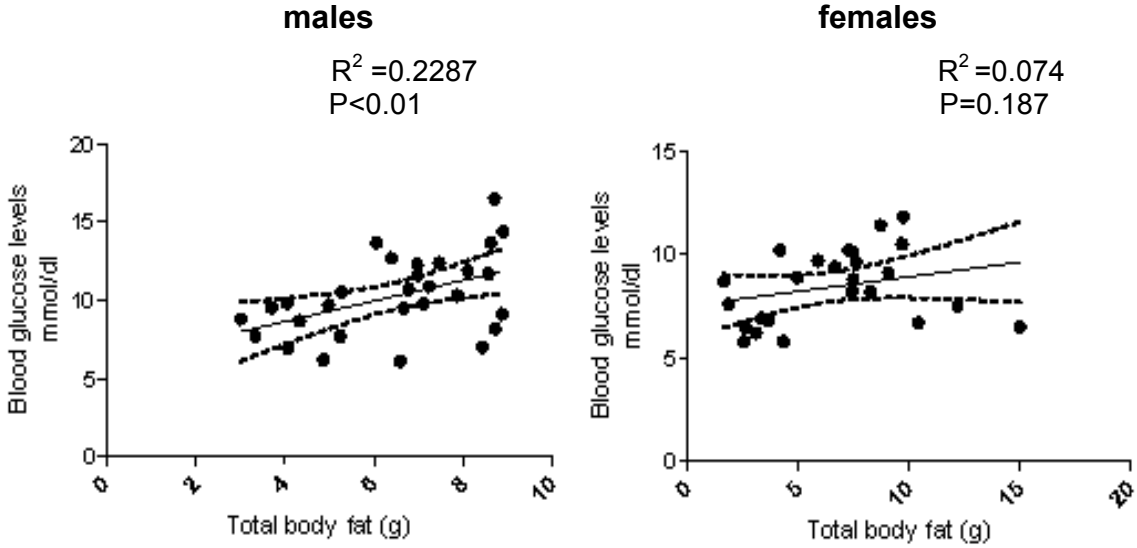


Figure 64. Correlation between total body fat and blood glucose levels in male and female offspring.

5.4.9.2 Total body fat and energy expenditure

Total body fat was negatively correlated with energy expenditure in male offspring ($p<0.0001$, see Figure 65). As total body fat increases, there is a corresponding reduction in energy expenditure. No significant correlation was evident in female offspring.

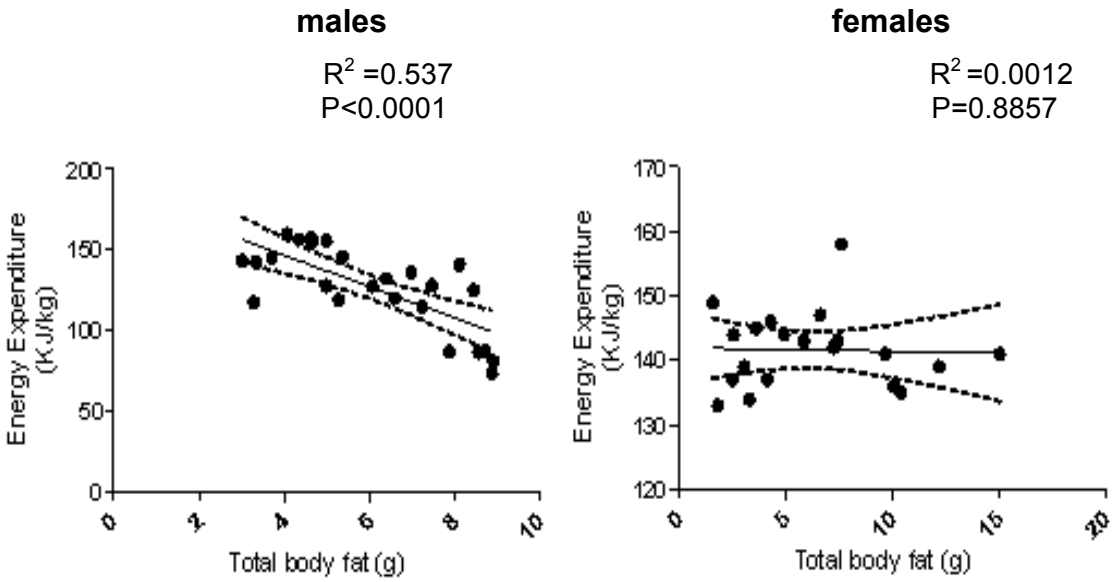


Figure 65. Correlation between total body fat and energy expenditure in male and female offspring.

5.4.9.3. Total body fat and intra-muscular fat deposition in male offspring

There was a positive correlation between total body fat and energy expenditure in male offspring ($p<0.0001$, see Figure 66). As total body fat increases, there is a corresponding increase in intramuscular fat.

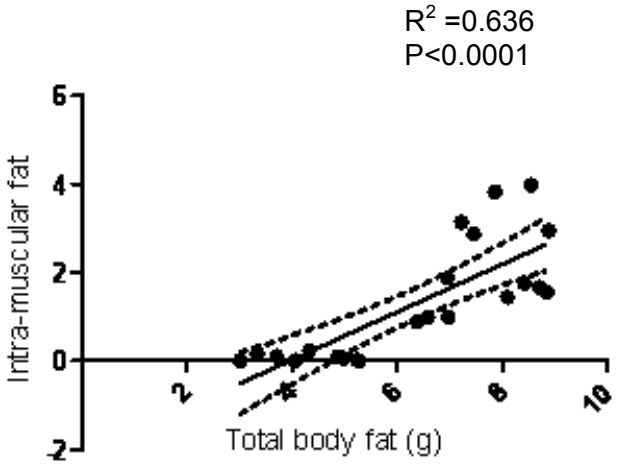


Figure 66. Correlation between total body fat and intra-muscular fat deposition in male offspring.

5.4.9.4 UCP-1 mRNA levels in iBAT and energy expenditure

UCP-1 mRNA levels in the iBAT was positively correlated with energy expenditure in male offspring ($p<0.01$). In the male offspring, as the mRNA levels for UCP-1 in the iBAT increases, so does the energy expenditure. However, this is not the case in the females where there is no correlation in UCP-1 mRNA levels with energy expenditure (Figure 67).

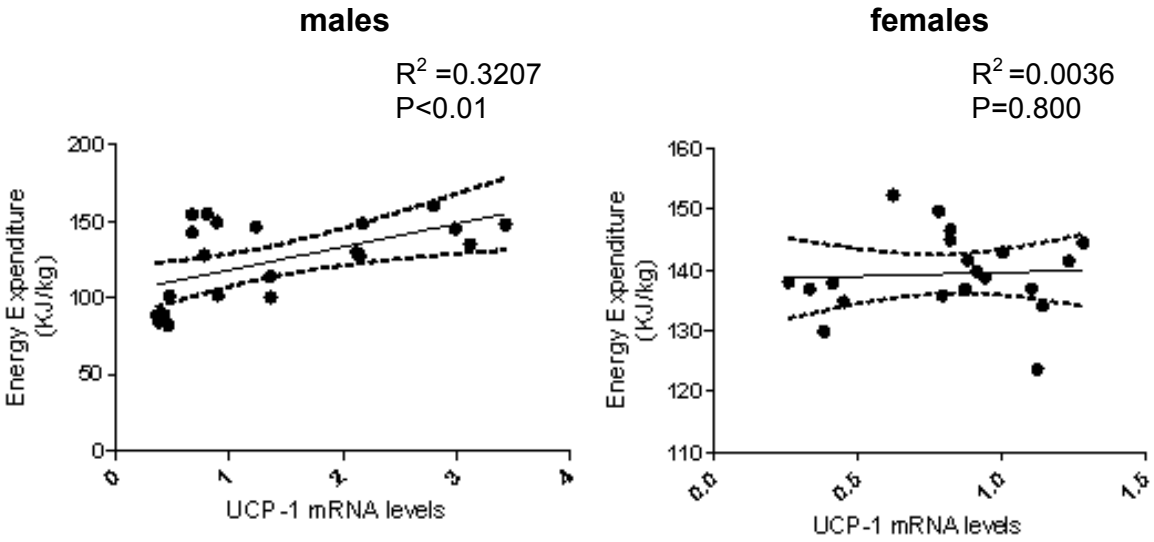


Figure 67. Correlation between UCP1 mRNA levels in iBAT and energy expenditure in male and female offspring

5.4.10. Maternal-offspring dietary interaction

In male offspring, there was significant maternal (protein-restriction) x offspring (HF) dietary interactions in total body fat, energy expenditure and systolic blood pressure at $p<0.001$, and intramuscular fat deposition at $p<0.0001$ (see Table 21). In females however, the only maternal x offspring dietary interaction that was found was that of systolic blood pressure ($p<0.001$). There was also an offspring dietary effect in both males and females for total body fat ($p<0.1$), systolic blood pressure ($p<0.001$) and intramuscular fat deposition in males ($p<0.01$). There were also maternal dietary effects in both males and females in systolic blood pressure ($p<0.0001$ and $P<0.01$, respectively).

Table 21. Results from 2-way ANOVA analyzing maternal x offspring dietary interactions for total body fat (TBF), energy expenditure (EE), systolic blood pressure (SBP) and intra-muscular fat deposition (IMF).

	Physiological parameters			
	TBF	**EE**	**SBP**	**IMF**
Maternal diet effect				
males	ns	ns	p<0.0001	ns
females	ns	ns	p<0.01	nd
Offspring diet effect				
males	p<0.01	ns	p<0.001	p< 0.01
females	p<0.01	ns	p<0.001	nd
Maternal x offspring diet effect				
males	p<0.001	p<0.001	p<0.001	p<0.0001
females	ns	ns	p<0.001	nd

ns = no significant difference, nd = no data

5.5. Discussion

The results in this Chapter show a reduction in body weight trajectory in male offspring that were exposed to greatest degree of dietary mismatch between prenatal and post weaning diets. Interestingly, these offspring had the greatest amount of body fat that had extended to fat accumulation in their skeletal muscle. This was accompanied by reductions in mRNA expression of UCP1 and β-3 adrenergic receptor in their iBAT. Furthermore, these PR/HF offspring had reduced energy expenditure. These findings suggest that these male offspring exposed to the greatest degree of prenatal - post weaning dietary mismatch have increased energy efficiency and propensity for greater energy storage and fat accumulation.

In this Chapter I also observed no changes in food intake and additionally no changes in food preference between offspring from both C-fed and PR dams during pregnancy and lactation. These offspring still preferred the high fat diet over the other diets high in protein or carbohydrates. This is different to what was reported in a previous study done in rats where there were differences in food preference between the offspring from PR dams to those from normal fed dams [276]. A possible reason for the difference between my study and this study done in rats was the window of exposure to maternal protein restriction. In the present study, maternal protein restriction was imposed during pregnancy and lactation, whereas the study done in rats restricted maternal dietary protein only during the pregnancy period. A preference for fat-rich food might be an appropriate response to maternal undernutrition, as it promotes the consumption of food that would provide the greatest amount of calories, which would be an advantageous adaptation to an energy-depleted nutritional environment. The absence of any changes in food preference is thus reflective of an inability to adapt appropriately, as offspring from dams that were protein-restricted during pregnancy alone might. This inability to alter food habit and intake observed in the animals from the present chapter might be attributable to the fact that if PR

is imposed during lactation, offspring brain development is still ongoing. This difference in window of exposure to maternal protein restriction could have different effects on the development of the limbic and regions of the frontal lobe of the brain which are responsible for the processing of taste and smell, as well as the hypothalamic regions involved in appetite regulation, which are still developing at the time of birth [277]. Moreover taste buds are not fully innervated by geniculate ganglion neurons until postnatal day 40 [278]. Thus, maternal protein restriction during the lactation period could therefore impact differentially on the development of the taste system, which could then affect the food preference of adult offspring differently compared to offspring from dams that were protein restricted only during the pregnancy period. Given that the human hypothalamus is already well developed and neural connections well established by the time of birth, unlike those of the mouse, the finding of changes in food preference when maternal protein-restriction was limited to pregnancy alone might be reflective of the human scenario [279].

Consumption of a high-fat diet leads to increase adiposity, which in turn could have elevated UCP-1 expression in the iBAT and increased adaptive thermogenesis to further prevent weight gain [280,281]. This was shown in the present study where there was significant increase in UCP-1 gene expression in the iBAT of the C/HF male offspring. On the other hand, exposure to maternal protein-restriction during pregnancy and lactation seems to prevent this increased expression of UCP-1 in the iBAT even in the high fat-fed male offspring. Another interesting finding in my study was that the PR/HF males were considerably lighter even when they had reduced energy expenditure and downregulation of genes that promote thermogenesis. Several studies have suggested that maternal undernutrition during early development leads to appropriate physiological changes in response to altered nutrition which serve to better-prepare offspring to the perceived postnatal nutritional environment [128, 130]. In one of these studies, it was suggested that altered thermogenesis may be a means by which offspring exposed to maternal global

undernutrition during pregnancy increase their energy efficiency [130]. The offspring from these studies showed postnatal catch-up growth when fed post weaning a HF diet [26]. In the present study however, offspring from protein restricted dams had lower body weight trajectory following post weaning HF feeding compared with HF-fed offspring from C-fed dams. This difference might be attributed to a number of factors. For instance, my study used maternal protein restriction as a proxy for undernutrition, whereas others have resorted to reducing the dam's total food intake. My results show that the lower body weight trajectory seen in offspring from PR dams is different from what I and others have observed in offspring from dams that were subjected to global undernutrition during pregnancy (see Chapter 3). This would suggest the importance of protein for muscle development and growth [282]. Although muscle fibre number and type are already fully established at birth in rodents, muscle growth during the lactation period occurs at a fast rate and therefore restricting protein availability during pregnancy and lactation would have detrimental effects on muscle growth and impact on the overall growth trajectory [283, 284]. This may also explain why no such changes in body weight trajectory were observed in offspring from dams that were protein restricted only during the pregnancy period (see Chapter 4). A previous study in rats where protein restriction was imposed during pregnancy and lactation also found similar reduction in the offspring's body weight trajectory compared to offspring from C-fed dams [271].

The observation that the PR/HF male offspring were lighter but fatter than the C/HF animals may be due to a reduction in their muscle mass. Although I did not measure muscle mass in the offspring in my study, others have previously demonstrated that maternal undernutrition during pregnancy resulted in offspring with reduced muscle mass due to ablated muscle tissue development [285, 286]. Moreover, it was also reported that extending maternal undernutrition through to lactation further impairs muscle growth in the offspring [287]. A reduction in muscle mass in turn could impact on metabolism and

hence energy expenditure. Energy expenditure has three major components, namely physical activity, obligatory energy expenditure and adaptive thermogenesis [106]. Obligatory energy expenditure accounts for the energy expended to carry out basic organ and cellular functions. This has been shown to vary according to body composition as different types of tissue have differing energetic requirements. The superior oxidative requirements of muscle tissue compared to other tissue types mean that it uses more energy and therefore contributes more than any other tissue to obligatory energy expenditure [106]. Variations in muscle mass have been shown to account for a significant degree of variation in energy expenditure within a population, with lower muscle mass associated with reduced levels of energy expenditure [288-290]. A reduction in muscle mass, in combination with decreased thermogenesis, will most likely contribute to the reduction in energy expenditure and increased energy efficiency, as was observed in the PR/HF offspring. This association is depicted in Figure 68.

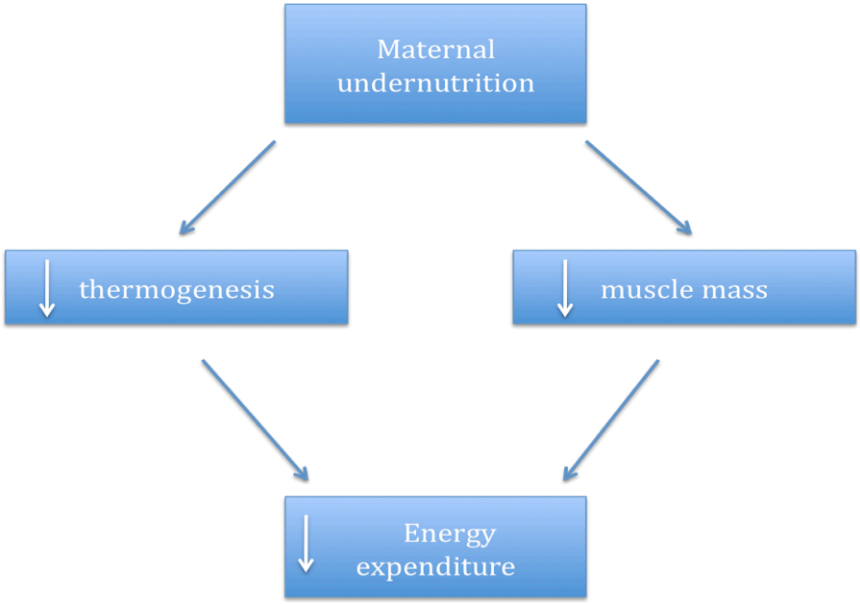


Figure 68. Diagrammatic representation of the contribution to reduced energy expenditure in male PR/HF offspring.

The reduction in energy expenditure in the PR/HF male offspring most likely contributed to the increased adiposity seen in these animals. In this Chapter, I found that there was a strong negative correlation between reduced energy expenditure with increasing adiposity in the PR/HF male offspring. All the HF-fed offspring also showed reduction in RQ values compared to the C-fed groups regardless of their sex or the the diet of their mother during pregnancy and lactation. It is interesting that while PR/HF males had increased fat depots, particularly gonadal and inguinal fat compared with the C/HF animals, the iBAT were similar in both group of offspring. Thus not only is there reduced UCP1 and β-3 adrenergic receptor expression in iBAT in male offspring exposed to the greatest degree of nutritional mismatch, but there is also no difference in the amount of iBAT that they have. Both of these factors are likely to contribute to the lower energy expenditure seen in PR/HF males.

Interestingly, while energy expenditure was correlated with UCP-1 mRNA levels and total body fat in male offspring, there was no such correlation in the females. This sex-related difference in the relationship between body fat, energy expenditure and UCP-1 mRNA

levels may be due to the influence of circulating sex steroids on uncoupling-protein activity [291,292]. This means that the normal feedback mechanism that allows the male offspring to increase thermogenesis when fed a hypercaloric diet may be influenced by circulating steroids in females [293]

A combination of consumption of a high fat diet and lower energy expenditure has been previously shown to increase adiposity [294]. The increased adiposity seen in PR/HF male offspring extended to fat accumulation in their skeletal muscle; that the increase in body fat is positively correlated to increased intramuscular fat deposition. There is evidence for elevated intra-muscular fat deposition in obese human which is secondary to the saturation of the storage capacity of adipose tissue [295]. There is intramuscular fat deposition in patients with lipodystrophy, where adipose tissue is absent and muscle tissue is used to store fat instead [296]. Evidence also suggests that as well as skeletal muscle, cardiac muscle is utilized as a storage depot for fat when adipose tissue is already saturated [297]. As cardiac muscle fat accumulation has been linked with heart disease and hypertension [297], this may contribute to the grossly elevated systolic blood pressure observed in PR/HF male offspring.

The results in this Chapter demonstrate a clear interaction between maternal undernutrition and offspring overnutrition to bring about sex-specific changes in energy efficiency and adiposity in the offspring. This is in agreement with the predictive-adaptive response theory, where a mismatch between maternal diet during pregnancy and the post weaning diet causes deleterious alterations in the physiology of the offspring. The consequences of intramuscular fat deposition are considerable. Studies in mice have demonstrated the causal relationship between the accumulation of fat in the muscle and insulin resistance [298,299]. While the presence of intramuscular fat deposition may not indicate insulin resistance, it certainly predisposes to its development. When the oxidative or storage

capacity of fat depots reaches maximum, harmful bi-products of lipid metabolism are created which appear to have a negative impact on insulin sensitivity and energy homeostasis [300-302]. It is hypothesized that the production of such metabolic bi-products is the culmination of a negative-feedback pathway that prevents excess energy storage and promote energy expenditure. Persistent production of such metabolic bi-products however, impairs insulin sensitivity particularly in times of nutritional abundance [302]. One such harmful bi-product of fat metabolism following saturation of adipose tissue is ceramide. This molecule has been shown to antagonize insulin signalling and mitochondrial function, not only in skeletal muscle but also in the heart and pancreas, leading to pathological consequences [303]. Therefore excess fat accumulation and altered metabolic function may contribute to cardiovascular dysfunction as well as insulin resistance. Ceramide may therefore be an important link between impaired energy expenditure and altered cardiovascular function, and potentially contributes to the grossly elevated systolic blood pressure observed in PR/HF male offspring.

While I found significant elevation in blood pressure in male PR/HF offspring compared to the C/HF offspring there was no significant differences in blood glucose levels between these offspring groups, The reason for this could be that PR/HF male offspring are compensating for increase fat accumulation and rising glucose levels by increasing insulin output from the pancreas, thus preventing the progression to insulin resistance and keeping blood glucose levels under control. In male, but not female offspring, blood glucose levels were strongly correlated with total body fat levels. A previous study has shown that females are more resistant to the fat-induced insulin resistance [180]. This may explain the differences in correlation between total body fat and glucose levels between the male and female offspring in my study.

In the present study, I found elevated systolic blood pressure in offspring from PR dams. This is in agreement with previous studies in which protein restriction during pregnancy elevated systolic blood pressure [91, 304]. By giving offspring from PR dams a HF diet there were further increases in their blood pressure over and above the level found in the PR/C animals. These results were similar to both male and female offspring, which is in contrast to what I found in terms of fat deposition and energy expenditure. Furthermore I also found a significant interaction between the maternal and offspring dietary challenges to bring about the greatest increase in blood pressure. The lack of sex differences with respect to cardiovascular phenotype compared to sex-dependent changes in the metabolic parameters most likely reflect the important influence of the sex steroids on organ systems involved in energy homeostasis. Estrogen, as previously mentioned is directly involved in energy expenditure regulation and influences the expression of uncoupling proteins in adipose tissue [174,248], hence the sex differences observed for adiposity and energy expenditure in the offspring. There is little evidence in mouse models to suggest, however that estrogen is directly involved in cardiovascular homeostasis.

Finally, the findings of maternal x offspring dietary interactions for intramuscular fat deposition, adiposity, energy expenditure and systolic blood pressure support the predictive-adaptive response theory.

Chapter 6

General Discussion

This thesis examined the role of early life nutritional environment and its interaction with the post weaning environment on the future health of the offspring. I have used two forms of maternal nutritional manipulations during early development; one is by reducing the total food intake of the dams during pregnancy, and the other is by reducing the protein content of the maternal diet, but maintaining its total caloric level, exclusively during the pregnancy period or during the pregnancy and lactation periods.

The purpose of comparing the effects of maternal global undernutrition by reducing the dam's food intake versus reducing only the protein content of the dam's diet without compromising the caloric content (i.e. isocaloric compared with control diet) was to determine the impact of the severity of the maternal undernutrition on the metabolic and cardiovascular phenotype of adult offspring. Furthermore, comparing the effects of maternal dietary protein restriction imposed exclusively during the pregnancy period versus extending the protein-restricted maternal diet to include the lactation period will elucidate the importance of window of exposure to a nutritionally deficient environment during early development on the offspring phenotype in later life.

I also examined the possible interaction of these maternal nutritional manipulations with manipulation of the offspring's diet from weaning to adulthood by feeding then a diet that is high in fat. This is to produce a dietary mismatch to test whether the Predictive Adaptive Response (PARs) phenomenon [20] occurs in this model of nutritional manipulations in mice. The PARs hypothesis suggest that the greater the environmental mismatch, the more likely will the offspring develop disease phenotypes including the metabolic syndrome [20].

6.1 Severity of *in utero* undernutrition

In this thesis, I found increased body weight trajectory in male offspring from dams that were subjected to global undernutrition during pregnancy compared to males from dams that received adequate nutrition during pregnancy, when both groups were fed from post weaning a standard chow diet. This increased body weight was not attributed to an increase in the offspring's caloric intake as they were similar in both groups of male offspring. Interestingly, I only observed this difference in growth trajectory in the male and not in female offspring. In addition to increased growth trajectory, these offspring exposed *in utero* to maternal food restriction had increased adiposity. This is in contrast to what we observed in offspring from dams that were protein-restricted during pregnancy where I did not find any difference in growth trajectory compared with offspring from dams fed the chow diet. Moreover these offspring exposed *in utero* to maternal protein restriction had similar amount of fat in their bodies compared to those from chow-fed dams. This was a novel finding and to my knowledge has not been documented before in the literature.

The indirect calorimetry results showed that the male offspring from food-restricted dams had reduced energy expenditure measured by indirect calorimetry. These changes were again not observed in the females. These results therefore suggest that maternal caloric restriction by global undernutrition, being the more severe form of *in utero* undernutrition, resulted in increased energy efficiency leading to increased fat accumulation. The sex-dependent reduction in energy expenditure in the male offspring, despite both male and female offspring from globally undernourished dams showing increased adiposity, could be attributed to differences in circulating gonadal steroid milieu in males compared to females. Several studies have reported a direct influence of hormonal changes associated with the oestrus cycle on energy expenditure, particularly affecting β-adrenergic control of energy expenditure regulation [305-308]. One of the possible mechanisms for the reduction in energy expenditure could be due to an impairment of thermogenesis, as

reflected by the reduction in UCP-1 mRNA levels in the intrascapular brown adipose tissue (iBAT). This notion is supported by a study in mouse offspring exposed to a similar *in utero* global undernutrition having blunted diet-induced thermogenesis [170]. In my work, I was only able to measure UCP-1 and β-3 adrenergic receptor mRNA levels in the iBAT in the maternal protein-restricted study and not in offspring from globally undernourished dams. Nevertheless, I found similar levels of gene expression for UCP1 and β-3 adrenergic receptor in the iBAT in both sets of offspring from dams that were protein-restricted and those from chow-fed dams, which substantiates my finding that there were no changes in energy efficiency and thus no difference in body weight trajectory in both sets of offspring. Future studies will be needed to determine the UCP-1 and β-3 adrenergic receptor mRNA levels in the iBAT in offspring from the globally undernourished dams to determine whether these genes involved in thermogenesis are indeed altered by *in utero* exposure to maternal global undernutrition.

In terms of the offspring systolic blood pressure response to *in utero* exposure to maternal nutritional manipulations, I found that both maternal protein restriction and global undernutrition resulted in significant elevation of the offspring's blood pressure. Unlike the sex-dependent responses of the body weight trajectory and energy efficiency to *in utero* exposure to these maternal nutritional manipulations, both male and female offspring had raised blood pressure readings. My results, together with the findings by others showing imbalances in other nutrients in the maternal diet such as methionine [309] and fat [310] can also result in elevated blood pressure in the offspring, suggests that *in utero* exposure to any form of maternal nutritional manipulation have substantial impact on the cardiovascular health of the offspring. Possible mechanisms involved may include the impairment in the glucocorticoid signalling pathway involving 11 β-hydroxysteroid dehydrogenase. A previous study where offspring exposed to maternal protein restriction *in utero* developed cardiovascular dysfunction in adulthood [311]. Several studies have

found alterations in the normal functioning of the hypothalamic pituitary adrenal (HPA)-axis in offspring exposed to maternal protein restriction during pregnancy [312, 313], suggesting increased sensitivity to glucocorticoids via the glucocorticoid receptors. Moreover, enhanced glucocorticoid signalling has been shown to promote hypertension [314]. While altered glucocorticoid signalling has been implicated in hypertensive offspring exposed to maternal protein restriction during pregnancy, impaired nephrogenesis and renin-angiotensin system dysfunction in the offspring have been reported in studies involving both maternal protein-restriction and global undernutrition during pregnancy [315-317].

It seems that increasing the severity of maternal undernutrition *in utero* leads to more profound changes in the metabolic phenotype, but not the cardiovascular phenotype. Figure 69 shows the comparison of various changes in metabolic phenotype between male offspring from mothers that were protein-restricted during pregnancy and those that were subjected to global undernutrition. Figure 69A shows that high fat (HF)-fed male offspring from globally undernourished dams exhibited increased adiposity compared to HF-fed offspring from dams that were *ad-libitum* fed. On the other hand, PR/HF offspring had a similar level of adiposity to HF-fed offspring from chow-fed dams. A similar observation can be made with male chow-fed offspring from globally undernourished and protein-restricted dams (Fig 69B), with those receiving the more severe form of undernutrition during pregnancy (i.e. global undernutrition) being significantly fatter than their counterparts from *ad-libitum* fed dams. This difference, however, was not observed with PR/C offspring, who had comparable levels of fat as the C/C group.

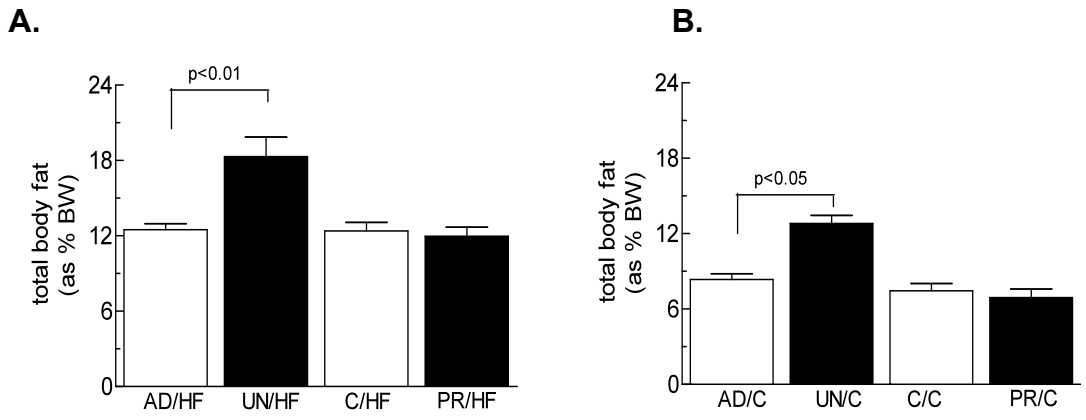


Figure 69. (A) Comparison of total body fat (as % of total body weight) in male high fat-fed offspring from dams that were globally undernourished (UN/HF) and those that were protein-restricted (PR/HF) during pregnancy. The comparison is based on the relative differences of these groups with their HF-fed counterparts that were from dams fed the chow diet *ad-libitum* (i.e. AD/HF & C/HF). (B) C comparison of total body fat between chow-fed offspring from globally-undernourished dams (UN/C) and those that were protein-restricted (PR/C) during pregnancy. The comparison is based on the relative differences of these groups with their chow-fed counterparts that were from dams fed the chow diet *ad-libitum* (AD/C & C/C).

I also observed elevated blood glucose levels in male UN/HF offspring compared to AD/HF offspring. However there were no differences in blood glucose levels between PR/HF offspring and C/HF offspring (Figure 70), again suggesting that severity of undernutrition during pregnancy has played an important role in altering the metabolic phenotype. The protective effect of hypophagia in male offspring exposed to maternal protein-restriction during pregnancy was not observed in offspring from globally undernourished dams compared to offspring from protein-restricted dams.

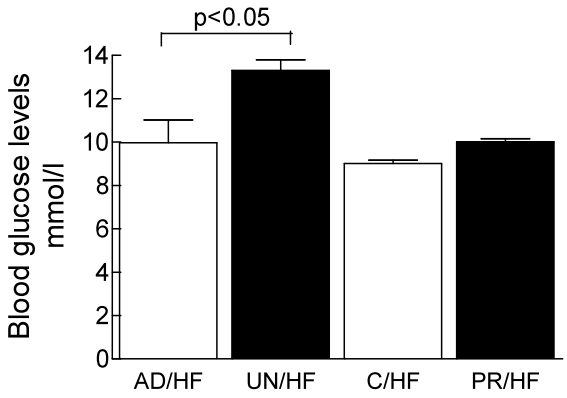
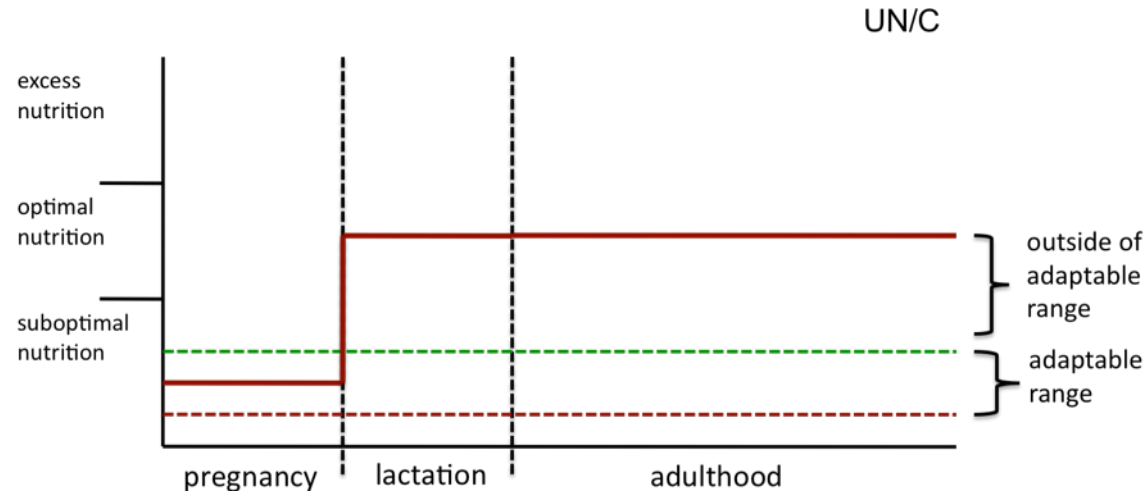


Figure 70. A comparison of the changes in blood glucose in HF-fed male offspring from globally undernourished dams (UN/HF) vs. HF-fed male offspring from dams fed the chow diet *ad-libitum* (AD/HF) and HF-fed male offspring from protein-restricted dams (PR/HF) vs. HF-fed male offspring from protein-restricted dams (PR/HF).

To help understand and explain the implications of the results of this thesis in the context of the predictive adaptive responses theory, I have developed a model (model of adaptable range) which explains how severity of maternal undernutrition during pregnancy, window of exposure to maternal undernutrition and presence of a post weaning HF impact on the health of the adult offspring. According to the model of adaptable range the extent or severity of maternal undernutrition has a bearing on how the offspring respond to post weaning nutrition and influences their ability to adapt to this nutritional plane without pathological or metabolic consequences. Increasing the severity of maternal undernutrition during pregnancy reduces the upper limit (dotted green line in Figure 71A) of the range of possible nutritional environments (adaptable range) to which offspring can adapt without adverse metabolic consequences. This increases the chances of offspring being unable to adapt successfully to a normal, let alone a plentiful nutritional environment in adulthood. For example in chow fed offspring from dams that were globally undernourished during pregnancy (UN/C), the actual nutritional environment experienced by adult offspring is outside of the range to which they can adapt, whereas the actual nutritional environment experienced by chow-fed offspring from protein-restricted dams lies within their adaptable

range (Figure 71B). This is because the upper limit of the adaptable range (green dotted line Figure 71A) of the UN/C offspring is lower than that for PR/C offspring in anticipation of similar severe form of postnatal undernutrition. This could explain the profound changes observed in terms of metabolic outcomes in the UN/C animals, compared to far fewer adverse metabolic outcomes exhibited by the PR/C animals.

A.



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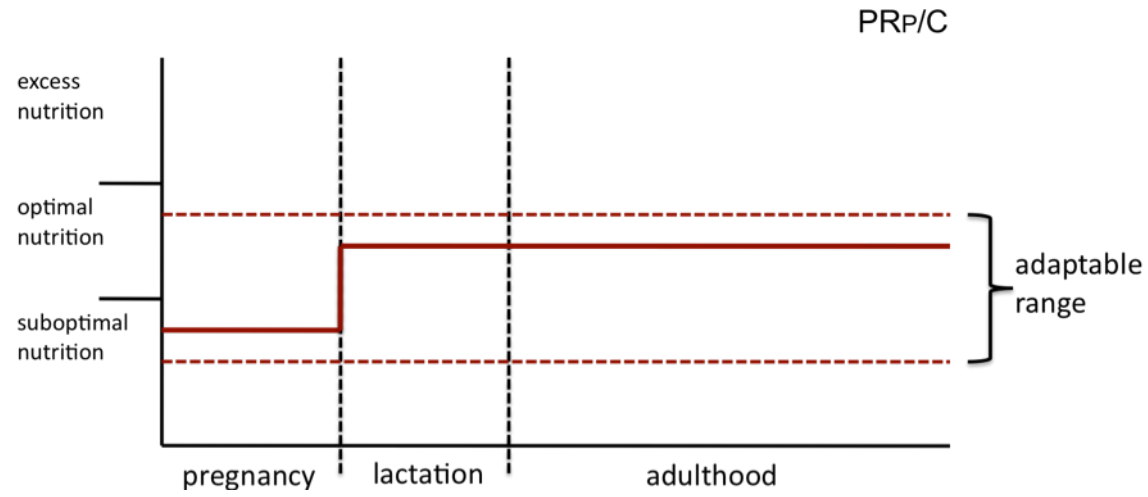


Figure 71. Model of adaptable range for (A) chow-fed offspring from dams that were globally undernourished during pregnancy (UN/C) and (B) chow-fed offspring from dams that were protein-restricted during pregnancy (PRP/C). The severity of undernutrition during pregnancy in the UN/C offspring means that the upper limit of the adaptable range is lower than PRP/C group.

6.2. Window of exposure to maternal undernutrition

From this point on in the discussion, I will refer to offspring from dams that were protein restricted *in utero* as PRP and offspring from dams that were protein restricted during pregnancy and lactation as PRPL. While my own study and others has demonstrated altered

cardiovascular and metabolic phenotype as a result of *in utero* undernutrition, the role of the early postnatal period, i.e. the lactation period, in developmental programming is less well understood. Previous studies have suggested that plasticity during the lactation period is vital for programming of offspring health. It has been shown that impairment of nephrogenesis following maternal protein restriction during pregnancy can be reversed by adequate nutrition during lactation in rats [63]. Another study also in rats has shown that the lactation period is more sensitive to protein restriction than the pregnancy period, at least in terms of altering the offspring's bodyweight trajectory [271].

I have found that PR$_{PL}$/HF offspring were lighter than C/HF offspring at the point of termination of the study whereas there was no difference in body weight between PR$_{P}$/HF offspring and C/HF offspring (Figure 72). Interestingly however, the PR$_{PL}$/HF offspring had accumulated more body fat compared to the HF-fed offspring from chow- fed dams (C/HF). This was observed in both the male and female PR$_{PL}$ offspring (Figure 73). This is in contrast to the PR$_{P}$/HF offspring that had the same amount of fat compared to the C/HF offspring, suggesting that extending the window of exposure to maternal undernutrition has more profound effects on the metabolic phenotype of offspring. The caloric intake and UCP-1 and β-3 adrenergic receptor mRNA levels in the iBAT in these PR$_{P}$ and PR$_{PL}$ offspring were similar compared with their respective control groups suggesting the difference in adiposity could not be attributed to either alteration in mechanisms the regulate food intake and energy efficiency. Changes in body composition in these offspring may be due to differences in their adiposity. Certainly reduced muscle mass in PR$_{PL}$ offspring might contribute to increased adiposity by altering whole body energy utilization as muscle is more metabolically active than other tissue types. Other factors influencing fat deposition that are unrelated to energy expenditure, such as cortisol levels, may also be involved producing this differences in adiposity observed between the two groups.

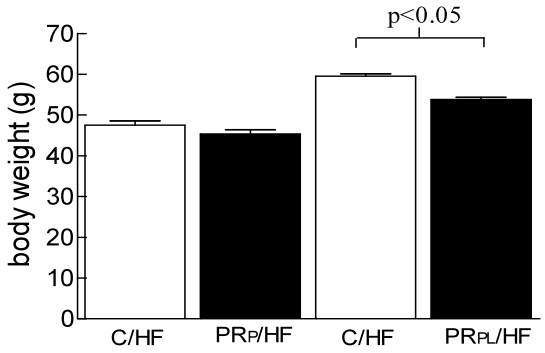


Figure 72. Comparison of body weight at the end of the study in HF-fed male offspring from dams that were protein-restricted during pregnancy (PRP/HF) offspring vs. HF-fed offspring from chow-fed dams (C/HF), and male HF-fed offspring from dams who were protein-restricted during pregnancy and lactation (PRPL/HF) vs. HF-fed offspring from chow-fed dams.

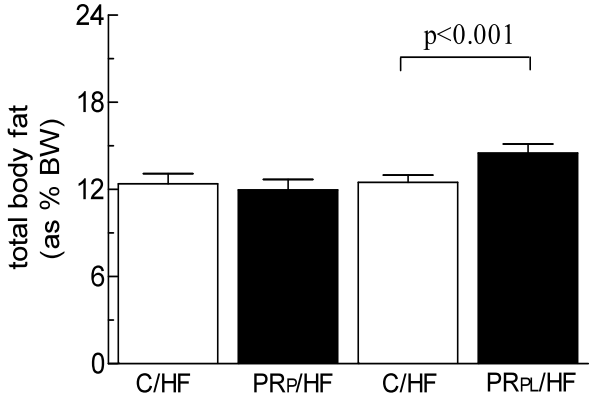


Figure 73. Comparison of total body fat in HF-fed male offspring from dams that were protein-restricted during pregnancy (PRP/HF) vs. HF-fed offspring from chow-fed dams (C/HF), and male HF-fed offspring from dams that were protein-restricted during pregnancy and lactation (PRPL/HF) vs. HF-fed offspring from chow-fed dams.

In terms of the offspring systolic blood pressure response to window of exposure to maternal protein restriction, there was very little difference between what was observed in offspring from dams that were protein restricted during pregnancy and those from dams that were protein-restricted during pregnancy as well as lactation. Both PRP and PRPL offspring had significant elevation in their blood pressure compared to their respective control groups (Figure 74).

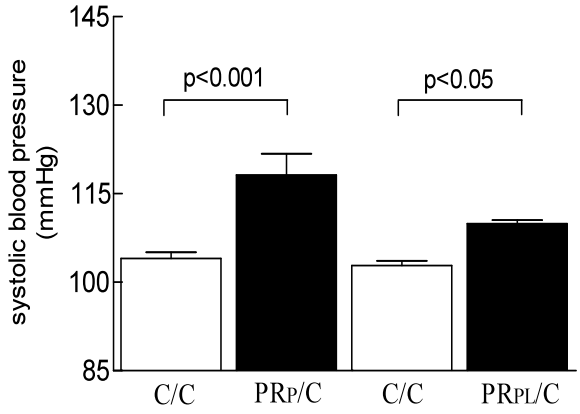


Figure 74. Comparison of systolic blood pressure in HF-fed male offspring from dams that were protein-restricted during pregnancy (PRP/HF) vs. HF-fed offspring from chow-fed dams (C/HF), and male HF-fed offspring from dams that were protein-restricted during pregnancy and lactation (PRPL/HF) vs. HF-fed offspring from chow-fed dams.

On the other hand however, I observed substantial differences in body weight trajectory and metabolic phenotype between offspring from dams which were protein restricted during pregnancy and those which received protein restriction during pregnancy and lactation. By extending protein restriction of the maternal diet to include the lactation period, I have found a reduction in bodyweight trajectory in the HF-fed male offspring (i.e. the PRPL/HF animals) . This is in contrast to what I have observed in offspring from dams that were exposed to protein restriction exclusively during pregnancy (PRP), where the bodyweight trajectory reflected more the post weaning nutrition, with both C.HF and

PR/HF offspring exhibiting similar body-weight trajectories irrespective of maternal diet. I observed a similar effect in female offspring, where the PRPL/HF offspring showed reduced body trajectory compared to C/HF female group; an effect which was not seen if maternal protein restriction was imposed only during pregnancy. In males there was no significant difference in body weight trajectory between PRPL/C and C/C, suggesting a combination of both maternal protein restriction during pregnancy and lactation as well as post weaning HF-feeding is required to bring about the reduction in body-weight trajectory in the offspring. The significance of a post weaning HF challenge in offspring previously exposed to maternal protein restriction *in utero* is further discussed below.

The results from this thesis confirm that the lactation period exerts a powerful influence on the phenotype of the offspring. The finding of more profound effects of maternal protein restriction during pregnancy and lactation on the offspring's body weight trajectory and adiposity, as opposed to the effects of maternal protein restriction exclusively during the pregnancy period, appears to suggest that restoring the diet of the protein-restricted dams to chow during lactation may offer some protection to the offspring from excessive body weight gain and increased adiposity, possibly though the reduction in their energy intake. If however maternal protein restriction was continuously imposed throughout the lactation period, these protective benefits were not observed.

According to the model of adaptable range, the early postnatal period, when the offspring are still nutritionally dependent on their mother's milk, provides a window of opportunity for the predicted nutritional environment to match the actual nutritional environment. I have therefore adopted the figure taken from a review by Gluckman and Hanson [264], suggesting that the range of nutrition to which an individual can adapt without adverse metabolic or cardiovascular consequences (grey area on graph) is dependent on its nutritional exposure during pregnancy (see Figures 75).

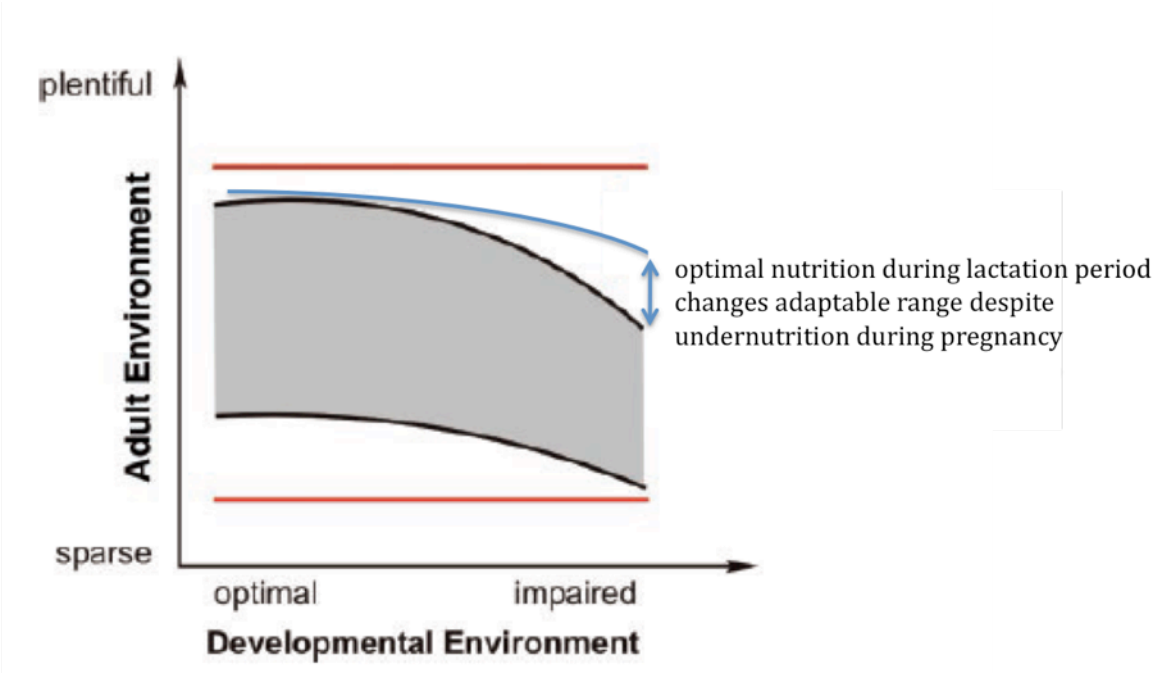
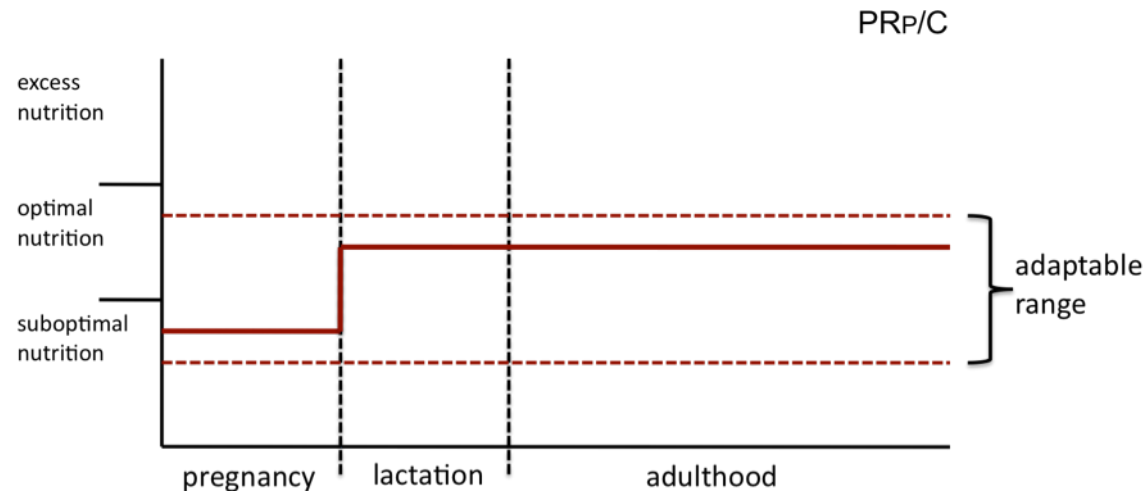


Figure 75. Adapted from Gluckman and Hanson [262]. The nutritional exposure during lactation increases the nutritional range to which an individual can adapt following previous undernutrition.

The results from my thesis suggest that the diet during lactation has a bearing on the eventual nutritional range to which the individual can adapt. This allows the upper limit of the nutritional range to increase if offspring were exposed to normal maternal nutrition during lactation, enabling them to survive within a greater range of nutritional variability. The actual nutritional environment experienced by the offspring exposed to maternal protein restriction exclusively during the pregnancy period would lie within the adaptable range (see Figure 76A). On the other hand, extending maternal protein restriction to include both the pregnancy and lactation period would reduce this adaptive range which may lessen the chances of the individual from surviving within a narrow range of nutritional variability (see Figure 76B). This will have serious consequences if one was to impose an unfavourable diet to the offspring after weaning, and is further discussed in Section 6.3.

A.



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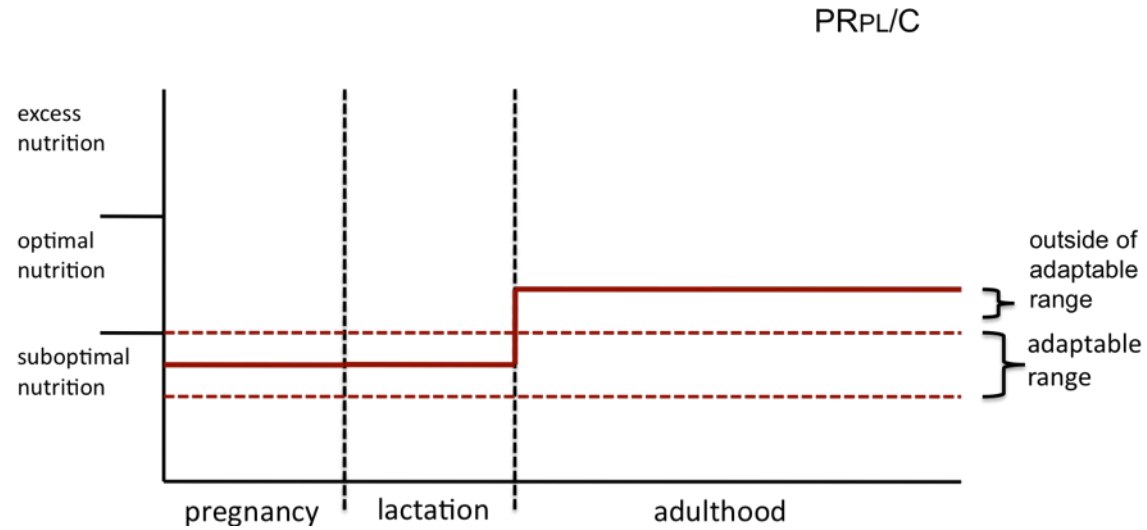


Figure 76. Model of adaptable range for (A) chow-fed offspring from dams that were protein-restricted during pregnancy (PRP/C) and (B) chow-fed offspring from dams that were protein-restricted during pregnancy and lactation (PRPL/C). Optimal nutrition during the lactation period allows PRP/C offspring to widen their adaptable range to nutrition. Thus their actual nutritional environment is within the adaptable range. This is not the case for PRPL/C offspring, where the actual nutritional environment during adulthood falls outside of the adaptable range, leading to greater disease risk and metabolic abnormalities.

6.3. Maternal-Offspring dietary mismatch

Several studies have suggested the necessity for hypercaloric post weaning nutrition to bring about the metabolic and cardiovascular changes following maternal undernutrition during pregnancy [26, 318]. In the maternal food restriction study, feeding the male offspring from post weaning a HF diet significantly increased body weight trajectory regardless of the nutrition of their mothers during pregnancy. Nevertheless, it was the HF-fed males from food restricted dams (i.e. the UN/HF group) that had the most amount of body fat and were hyperglycaemic. The increase adiposity in these UN/HF males may be linked to their low energy expenditure levels and suggests that they have become more efficient in storing fat. In the female offspring, it was only those from dams that were on normal chow diet that had significantly increase body weight trajectory following post weaning HF feeding (i.e. the AD/HF group). Unlike in males, post weaning HF feeding did not increase body weight trajectory in females exposed *in utero* to maternal food restriction (i.e. the UN/HF group). Nevertheless, the UN/HF female offspring exhibited the greatest adiposity compared to the other experimental groups suggesting that they have also become more efficient in storing fat. These metabolic adaptations which promote greater energy storage may not necessarily be beneficial during fetal development, but may provide sufficient survival capabilities, such as allowing for greater fat storage in a nutrient-depleted nutritional environment, if the perceived postnatal nutritional environment is correct. If however the actual postnatal environment differs from what is predicted, health problems may occur in later life.

Changing the type of maternal nutritional manipulation has a bearing on the response of the offspring to post weaning HF feeding in terms of their phenotypic outcomes. Although HF feeding resulted in similar increases in body weight trajectory in offspring from dams that were either food restricted or protein-restricted during pregnancy, there were differences in mechanisms as to how this phenotypic condition came about. Nonetheless,

the resulting increases in body weight by post weaning HF feeding to offspring from both food-restricted (i.e. the UN/HF animals) and protein-restricted dams (i.e. the PR/HF group) may be attributed to increased energy efficiency (i.e. by reducing energy expenditure or active thermogenesis). However, the accompanying adiposity in the UN/HF offspring was over and above the amount found in HF-fed offspring from dams fed the chow diet *ad-libitum* (i.e. the AD/HF animals). This suggests a synergistic effect of the *in utero* exposure to maternal food restriction and post weaning HF feeding. On the other hand, the increased body weight in the PR/HF offspring was mainly due to feeding them post weaning a HF diet. These differences may be due to the effect of the type of maternal nutritional manipulation imposed *in utero* on mechanisms that regulate their food intake. Food intake in the UN/HF animals was similar to the AD/HF group; whereas the PR/HF offspring, particularly the males, had reduced food intake compared with the HF-fed animals from chow-fed dams (i.e. the C/HF animals). Thus in the PR/HF males, there is an active attempt to control the rate of excess adiposity by significantly reducing food intake.

The nutritional status of the dams during lactation also has implications on the response of the offspring to post weaning HF feeding. Whereas HF-fed offspring from dams that were protein restricted exclusively during the pregnancy period (i.e. the PR$_{P}$/HF animals) shows similar increases in body weight gain compared with the HF-fed offspring from chow-fed dams (i.e. the C/HF group), those that were from dams that were protein-restricted during pregnancy and lactation (i.e. the PR$_{PL}$/HF animals) had lower body weight trajectory than the corresponding C/HF group. This was accompanied by increase adiposity in these PR$_{PL}$/HF offspring, particularly in the males, which was over and above the amount found in C/HF group. This suggests that, like in offspring from food-restricted dams, a synergistic effect of the *in utero* exposure to maternal protein restriction and post weaning HF feeding. This observed increased adiposity was also found in the skeletal tissues. The contributing mechanism for this increased adiposity may be due to increased energy

efficiency (i.e. by reducing active thermogenesis), similar to the UN/HF and PRP/HF offspring. However, like the UN/HF animals, this was not accompanied by changes in their food intake, which is probably why they have far greater adiposity than the C/HF group.

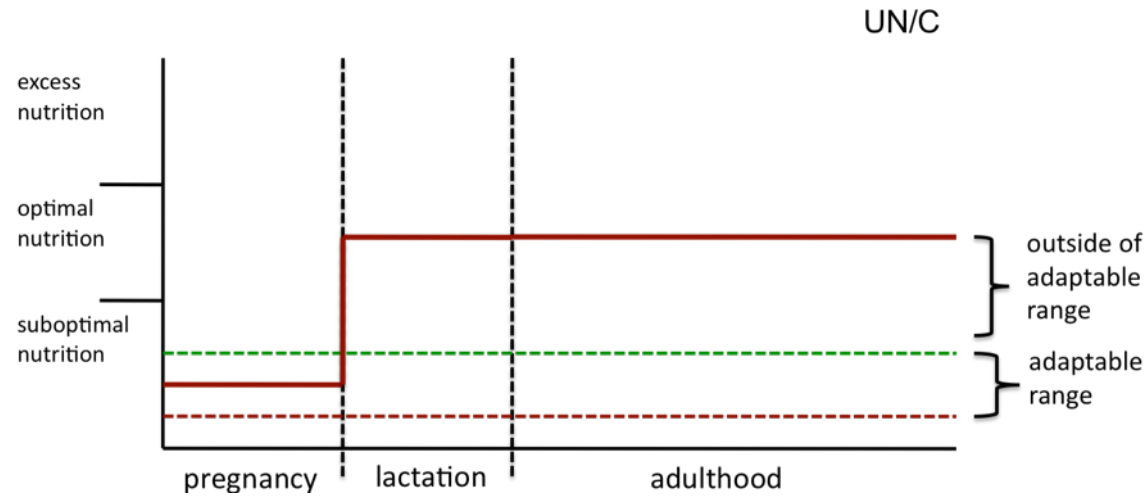
Many of the observed changes in adiposity, body-weight trajectory and expression levels of genes involved in thermogenesis in iBAT due to the greatest degree of prenatal-post weaning dietary mismatch were found to be greater in males rather than females. These sex-specific differences in terms of metabolic phenotype are likely due to differences in the levels of circulating sex hormones, given for example, that estrogen has been shown to promote greater fat deposition in female rodents [174].

My results are in agreement with the predictive adaptive response hypothesis [20] emphasizing the importance of the degree of nutritional mismatch and not just the nature of the maternal undernutrition or post weaning nutrition *per se* on the health of the adult offspring. It is worth mentioning that the HF diet used in this study, contains a high n-6/n-3 PUFA ratio which means it is high in saturated fats and low in essential-fatty acids,. Evidence suggests that consumption of a fatty diet with a high n-6/n-3 PUFA ratio such as the one utilized in the present study, causes greater metabolic alterations than diets with the same amount of overall fat content but with a lower n-6/n-3 PUFA ratio [319]. This may be important in producing some of the metabolic alterations observed in offspring exposed to the dietary mismatch. The present study suggests that much of the deleterious effects of post weaning HF feeding are due to the mismatch with maternal nutrition during early development.

Figure 77 demonstrates how maternal undernutrition during pregnancy followed by a HF post weaning diet in offspring could lead to an adaptation to a nutritional environment that they are ill-equipped to deal with. The extent to which the actual nutritional environment

experienced by adult offspring lie outside of the nutritional environment to which offspring can adapt without adverse metabolic or cardiovascular consequences is greater in the HF-fed than chow-fed offspring from dams that were undernourished during pregnancy.

A.



B.

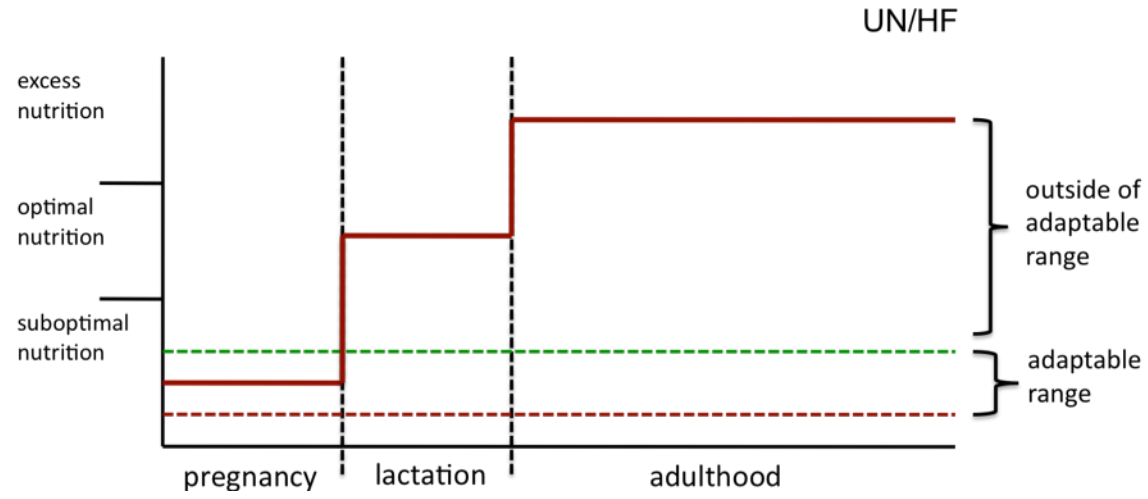


Figure 77. Model of adaptable range for (A) chow-fed offspring from dams who were undernourished by way of a 50% global food restriction during pregnancy (UN/C), and (B) HF-fed offspring from dams who were undernourished by way of a 50% global food restriction during pregnancy (UN/HF).

The studies on mismatched maternal and offspring diets have also provided some novel findings in terms of the cardiovascular outcomes in the offspring. Regardless of the nutritional manipulations imposed on the dams and window of exposure to these manipulations, this has always lead to significant elevation in the offspring's blood pressure. However, I only observed a further rise in blood pressure following post weaning HF feeding, over and above the effect of maternal dietary manipulation, in offspring exposed to maternal dietary protein restriction during pregnancy or during both pregnancy and lactation. This suggests that maternal undernutrition, regardless of severity or window of exposure seems to have a negative impact on cardiovascular function. Consumption of a normal chow diet during lactation appears to have no effect on cardiovascular function, perhaps because the heart is relatively better developed than other organs at the time of birth [320]. It appears that metabolic changes such as adiposity, body-weight trajectory, energy expenditure and appetite regulation are more likely to be influenced by the severity of undernutrition or window of exposure to maternal undernutrition than cardiovascular phenotype.

The overall results of this thesis have important implications particularly for individuals in countries wherein economic transition is occurring at a rapid phase, such as India, China and Brazil. Although economic prosperity means that the standard of living and healthcare also increases, some of the lifestyle changes that accompany prosperity such as increased consumption of diets that are high in fat can have deleterious consequences on their health. Thus any health initiatives to prevent the ever-increasing metabolic and cardiovascular health problems in these countries should focus on improving not on the health and lifestyle of the individual but should equally target the health and lifestyle of the mothers.

6.4. Future studies

Following my work, the following could be addressed in future studies:

1) Assess the effects of maternal protein restriction during exclusively during the lactation period on the metabolic and cardiovascular outcomes of the offspring. This will help to determine whether the effects observed in offspring from protein-restricted dams are due to a combination of undernutrition during the lactation period and pregnancy periods or due solely to the effects of maternal undernutrition during the lactation period.

2) Analyze changes in genes and biochemical components in the blood that are associated with hypertension. Analysis of genes such as nitric oxide synthase in peripheral arteries and β-1 adrenergic receptors in cardiac tissue, which have been shown to be altered in models of hypertension in rodents [321, 322] might help to establish a mechanism of altered systolic blood pressure and programming of cardiovascular dysfunction in the offspring.

3) Assess whether the changes in β-3 adrenergic receptor and UCP1 levels are accompanied by changes in the levels of circulating norepinephrine. Norepinephrine is required for activation of β-3 adrenergic receptor, thus increased levels of this hormone could influence energy expenditure regulation.

4) Assess whether there are changes in sympathetic nervous outflow to iBAT in offspring with reduced levels of β-3 adrenergic receptor and UCP1 in iBAT. This might establish a link between the periphery and central mechanisms regulating adaptive thermogenesis. This experiment can be achieved by retrograde tracing as described previously [323].

5) Analyze plasma levels of factors that could establish the mechanistic link between cardiovascular and metabolic observations. Such factors include plasma cholesterol levels as well as plasma C-reactive protein (CRP) levels.

Appendix 1: Publications

1. **Sellayah D**, Sek K, Anthony FW, Hanson MA, Cagampang FR (2008) Sensitivity of housekeeping genes in the hypothalamus to mismatch in diets between pre- and postnatal periods in mice. *Neuroscience Letters* 477:54-57 [Epub 2008 Sept 27].

2. **Sellayah D**, Sek K, Anthony FW, Watkins AJ, Osmond C, Fleming TP, Hanson MA, Cagampang FR (2008) Appetite regulatory mechanisms and food intake in mice are sensitive to mismatch in diets between pregnancy and postnatal periods. *Brain Research* 1237:146-52. [Epub 2008 Aug 13].

Appendix 2: Manuscripts in preparation

1. **Sellayah D**, Terroni PL, Anthony FW, Watkins AJ, Coen CW, Ghatei MA, Osmond C, Fleming TP, Hanson MA, Cagampang FRA. Mismatch between maternal diet during pregnancy and post-weaning offspring diet alters energy efficiency and cardiovascular function in mice.

2. **Sellayah D**, Anthony FW, Asopa S, Watkins AJ, Fleming TP, Ohri SK, Hanson MA, Cagampang FRA Maternal protein restriction during pregnancy and lactation increases susceptibility of adult mouse offspring to developing obesity and cardiovascular dysfunction following post weaning high fat feeding.

3. **Sellayah D**, Anthony FW, Watkins AJ, Fleming TP, Hanson MA, Cagampang FRA. Window of exposure to maternal dietary protein restriction influence food choice in the adult mouse offspring.

4. Bruce KD, **Sellayah D**, Anthony FW, Wang C, Hanson MA, Cagampang FRA, Byrne CD. A high unsaturated fat, high protein and low carbohydrate diet during pregnancy and lactation modulates hypothalamic and peripheral adipokine receptor gene expression in adult mouse offspring.

Appendix 3: Abstracts of presentation at school, national and international scientific meetings

1. Cagampang FR, **Sellayah D**, Anthony FW, Watkins AJ, Fleming TP, Hanson MA (March 2009) Maternal protein restriction during pregnancy and lactation increased susceptibility of mouse offspring to developing obesity and cardiovascular dysfunction following post-natal high fat feeding. Abstract published in *Reproductive Sciences* 16:322 (Poster presentation at the 2009 Society for Gynecologic Investigation Annual Scientific Meeting**;** Glasgow, UK).

2. **Sellayah D**, Anthony FW, Watkins AJ, Fleming TP, Hanson MA, Cagampang FR (September 2008) A mismatched pre- and post-weaning diet has window of exposure- and sex-specific effects on energy homeostasis, adiposity and cardiovascular function in mice. Abstract published in *Proceedings of the Physiological Society* 12: C10 & PC20 (Oral and poster presentation at the Physiological Society's themed meeting on Metabolism and Endocrinology; Oxford, UK).

3. **Sellayah D** (June 2008) A mismatched prenatal and post weaning diet has sex- and window of exposure-specific effects on offspring growth, food intake, systolic blood pressure and metabolism in mice. (Oral presentation at the Faculty of Medicine, Health and Life Sciences Postgraduate Conference, University of Southampton, Southampton, UK).

4. Cagampang FR, **Sellayah D**, Anthony FW, Hanson MA (May 2008) Mismatched prenatal and post-weaning diet has sex-specific effects on hypothalamic expression of genes involved in the regulation of energy intake in adult mouse offspring. (Poster presentation at the Multidisciplinary Workshop on Nutrition, Brain Development and Aging: Genetics, Epigenetics, and Behaviour; Kannapolis NC, USA).

5. **Sellayah D**, Anthony FW, Hanson MA, Cagampang FR (November 2007) Mismatched prenatal and post-weaning diet leads to sex-specific changes in expression of genes involved in the regulation of appetite and metabolism in the adult mouse offspring. Abstract published in *Early Human Development* 83:S131-132 (Poster presentation at the 5th World Congress on Developmental Origins of Health and Disease; Perth, Australia).

6. **Sellayah D** (June 2007) Effect of prenatal and post weaning dietary 'mismatch' on susceptibility of the adult offspring to the metabolic syndrome. (Poster presentation at

the Faculty of Medicine, Health and Life Sciences Postgraduate Conference, University of Southampton, Southampton, UK). *Was adjudged special mention as in the poster presentation at the conference*.

7. Bruce KD, **Sellayah D**, Barnet-Lamb E, Anthony FW, Wang C, Hanson MA, Cagampang FRA, Byrne CD (September 2006) A high unsaturated fat, high protein, and low carbohydrate diet during pregnancy and lactation modulates peripheral adipokine receptor gene expression in adult mouse offspring. Abstract published in *Early Human Development* 82:494-495 (Poster presentation at the 4th World Congress on Developmental Origins of Health and Disease; Utrecht, The Netherlands).

8. **Sellayah D**, Bruce KD, Wang C, Anthony FW, Hanson MA, Byrne CD, Cagampang FR (September 2006) High fat and protein diet during pregnancy and lactation alters hypothalamic adiponectin receptor gene expression in adult mouse offspring. Abstract published in *Early Human Development* 82:503-503 (Poster presentation at the 4th World Congress on Developmental Origins of Health and Disease; Utrecht, The Netherlands).

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