Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy.

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Abstract

Epidemiological associations between birthweight and cardiovascular disease in adult life are supported by rodent experiments showing that undernutrition in fetal life programmes adult blood pressure. In rats, the feeding of a maternal low protein (MLP) diet during gestation programmes hypertension. Given interest in the mechanistic role of epigenetic modification of gene expression in programming, this study aimed to assess the potential for a nutritional insult to impact across several generations. Pregnant female Wistar (F0) rats were fed a control (n=10) or MLP diet (n=10) throughout gestation. At delivery all animals were fed the same standard laboratory chow diet. At approximately 10 weeks of age, F1 generation offspring were mated to produce a second generation (F2) without any further dietary change. The same procedure was adhered to, to produce the F3 generation. Physiological analysis confirmed F1 generation MLP exposed offspring exhibited raised ($P<0.001$) systolic blood pressure and reduced nephron number ($P<0.001$) compared with controls. Raised blood pressure and reduced nephron number were also noted in the F2 generation ($P<0.001$) and this intergenerational transmission occurred via both the maternal and paternal lines, No effect was noted in the F3 generation.

Microarray analysis highlighted a number of genes that were differentially expressed however upon RT-PCR analysis results were not significant. DNA methylation analysis noted in a trend towards hypomethylation in MLP exposed rats and their offspring as described in previous studies.

In conclusion, data within this thesis shows for the first time, that fetal protein restriction may play a critical role in determining blood pressure and overall disease risk in a subsequent generation. It is clear from the data that both males and females

can transmit their phenotype to a subsequent generation. This finding suggests that maternal diet can influence the nature of epigenetic markers in germ line cells.

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Declaration

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

Matthew Harrison

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Publications arising from this thesis

Peer-Reviewed Papers

- Harrison MJ & Langley-Evans SC. (2008) Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. *British Journal of Nutrition*. 9 1-11.

Conference Abstracts

- Nutrition Society – Nottingham UK, July 2008. Intergenerational programming of nephrogenesis and hypertension following feeding of a low-protein diet in rat pregnancy.

- DOHaD – Perth Western Australia, November 2007. Intergenerational programming of blood pressure in rats following maternal protein restriction during pregnancy.

- DOHaD – Perth Western Australia, November 2007. Intergenerational programming of a lean phenotype in rats following maternal protein restriction during pregnancy.

- EARNEST – Budapest Hungary, April 2007 Intergenerational programming of blood pressure in rats following maternal protein restriction during pregnancy.

Abbreviations

11β-HSD 2	11β-hydroxysteriod dehydrogenase 2
ANOVA	Analysis of variance
ATP III	Adult Treatment Panel-III
B	Breeders
BAT	Brown adipose tissue
BMR	Basal metabolic rate
CHD	Coronary heart disease
CO_2	Carbon dioxide
CON	Control Protein
COV	Coefficient of variation
CVD	Cardiovascular disease
DNMT-1	DNA Methyltranferase 1
DNMT-3b	DNA Methyltranferase 3b
ECGF1	Endothelial cell growth factor 1
EDNRA	Endothelin receptor type A
FOA	Food and Agriculture Organisation
FSA	Food Standards Agency
GFR	Glomerular filtration rate
GR	Glucocorticoid receptor
GZMB	Granzyme B
HF	High Fat
HPA	hypothalmic-pituitary-adrenal
IGF2	Insulin-like growth factor 2
Il-7	Interleukin 7
IUGR	Intrauterine growth retardation
MA	Microalbuminuria
MJ/Kg	megajoules per kilogram
MLP	Maternal low protein
MMLV	Moloney Murine Leukemia Virus
MMP9	Matrix metallopeptidase 9
MUFA	Monounsaturated fatty acids
NC	Nutritional Challenge
NCEP	National Cholesterol Education Program
NIH	National Institutes of Health
P value	Probability
PBS	Phosphate-buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PPaα	Peroxisome proliferator activated receptor α
PUFA	Polyunsaturated fatty acids
RAS	Renin-angiotensin system
RMR	Resting metabolic rate
SD	Standard deviation
SEM	Standard error of mean
SPSS	Statistical package for social sciences
SREBP-1c	Sterol regulatory element binding protein 1c
TEE	Total energy expenditure
TM	Melting temperature

UCP1	Uncoupling protein 1
UK	United Kingdom
v/v	Volume per volume
w/w	Weight per Weight
WHO	World Health Organisation

1.0 General Introduction- Cardio-Vascular Disease

1.1 Introduction

An estimated 17.5 million people world-wide died from cardiovascular disease (CVD) in 2005. This represented 30% of all deaths. Approximately 80% of these deaths occurred in low- and middle-income countries. If the current trend of increasing CVD prevalence is allowed to continue, an estimated 20 million people per annum will die from CVD by 2015 (WHO, 2005).

The World Health Organisation (WHO) describes cardiovascular disease as being a chronic, non-communicable disease affecting the heart or blood vessels (WHO, 2005). The seriousness of CVD stems primarily due to the personal level of suffering endured by those affected and also from the spiralling costs associated with the disease and its associated conditions. CVD, places a heavy burden on the economies of many countries. In 2004, CVD cost the United Kingdom (UK) economy £29.1 billion with cerebrovascular disease and coronary heart disease (CHD) accounting for 56% (£16.5 billion) of the total (Luengo-Fernandez *et al.*, 2006). It has been estimated that between 2006 and 2015, China will lose $558 billion in foregone national income due to heart disease, stroke and diabetes (WHO, 2005).

Cardiovascular disease is the generic name for a group of diseases that affect the heart and the blood vessels (WHO, 2005). These include,

- Coronary heart disease – disease of blood vessels supplying the heart muscles.
- Cerebrovascular disease – disease of the blood vessels supplying the brain.

- Peripheral arterial disease – disease of blood vessels supplying the limbs.
- Rheumatic heart disease – damage to the heart muscle and heart valves from rheumatic fever (an autoimmune inflammatory disease that develops after infection with streptococcal bacteria).
- Congenital heart disease – malformations of the heart structure.
- Deep vein thrombosis – blood clots in the leg veins which may dislodge and translocate to the heart or lungs.
- Hypertension – A blood pressure greater than a systolic blood pressure of 140 mmHg, or a diastolic blood pressure of 90 mmHg or over.

Coronary heart disease, cerebrovascular disease and peripheral arterial disease are common problems associated with chronic CVD. These conditions are all manifestations of atherosclerosis, which is the process where plaques build up on the inner surface of arteries. These plaques later harden and restrict blood flow through the effected arteries. Plaques may also break away from the surface of the arteries, becoming lodged in the arteries supplying the heart or the brain and subsequently causing heart attacks or strokes (WHO, 2005).

1.1.1 CVD Risk Factors

To date over 300 separate risk factors have been associated with the development of CVD. In the developed world over 30% of all CVD is attributed to five main risk factors; namely high blood pressure, obesity, high circulating cholesterol and tobacco and alcohol use (WHO, 2005). These factors also rank

highly in developing countries with low mortality, such as China, and developing countries with high mortality, such as India, where the populations also face the burden of communicable diseases and undernutrition (WHO, 2005). The main risk factors can be characterised into three different categories, major modifiable risk factors, other modifiable risk factors and non-modifiable risk factors (Table 1.1)

Table 1.1 Summary of CVD risk factors (WHO, 2005).

Major modifiable risk factors	**Other modifiable risk factors**	**Non-modifiable risk factors**
High blood pressure	Low socioeconomic status	Advancing age
Dyslipidaemia	Mental ill-health	Genetics
Tobacco use	Psychosocial stress	Gender
Physical inactivity	Alcohol use	Ethnicity/ race
Obesity	Use of certain medication	
Elevated homocysteine	Lipoprotein(a)	
Diabetes mellitus	Left ventricular hypertrophy	

Considerable health benefits can be achieved by increasing physical activity and reducing alcohol and tobacco usage combined with eating a “healthy diet”. In the United Kingdom (UK) the Food Standards Agency (FSA) classifies a “healthy diet” as a diet consisting of a third of fruit and vegatables which should include at least five 80g portions of fruit and vegetables per day, and a third starchy foods such as potatoes, wholegrain breakfast cereals and lentils. The final third of the diet should consist of a mixture of dairy foods such as milk and cheese, and non-dairy sources of protein like meat and fish. Consumption of foods and drinks rich in fat and sugar should be kept to the mimium. This has been summerised in the much publicised FSA “eatwell plate” (Figure 1.1).

Figure 1.1 The Eatwell plate (FSA, 2000)

1.1.2 Metabolic syndrome

The metabolic syndrome is a combination of metabolic and physical abnormalities that increase the risk of developing CVD. Although the concept of the metabolic syndrome, also known as Syndrome X (Reaven, 1988), has existed since the 1920's when a Swedish physician, Kylin (1923) described the syndrome as the clustering of hypertension, hyperglycaemia, and gout, (Kylin, 1923), it was not until 1988 that there was an attempt to achieve an agreement on an internationally recognised definition in order to provide a tool for researchers and clinicians. The World Health Organisation (WHO, 2005) proposed that in order to be diagnosed with the metabolic syndrome an individual must have diabetes or impaired fasting glycaemia or impaired glucose tolerance or insulin resistance, plus 2 or more of the following criteria:

- Hypertension: blood pressure> 140/90 mm Hg
- Microalbuminuria: albumin excretion > 20µg/min

- Obesity: BMI >30 or a waist to hip ratio >0.9
- Dyslipidaemia: triglycerides >1.7mmol/L or HDL cholesterol <0.9mmol/L

Subsequently the National Cholesterol Education Programs Adult Treatment Panel III (NCEP:ATPIII) and the European Group for the Study of Insulin Resistance have formulated different definitions (Ferrannini, 1997; NIH, 2001; WHO, 2005). All three definitions agree on essential components; hypertension, obesity, glucose intolerance and dyslipidaemia. However they do differ in detailed criteria (Table 1.2). It is important to note that insulin resistance is very much at the centre of the metabolic syndrome and is tied into the spectrum of CVD risk factors, this is summarised in Figure 1.2.

Figure 1.2 CVD risk factors.

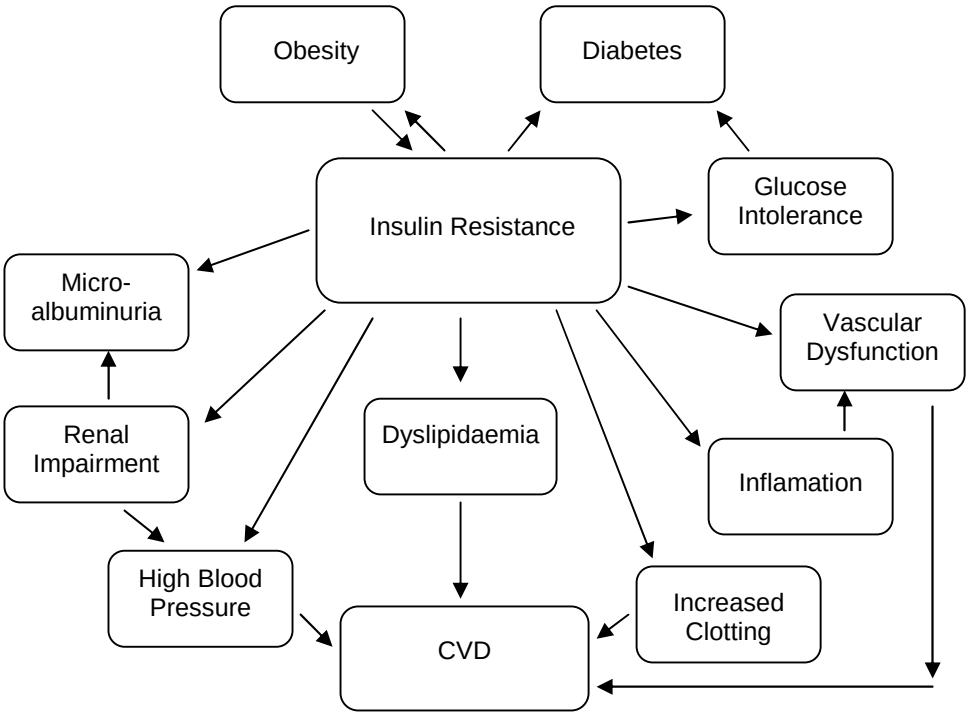


Table 1.2 Overview of metabolic syndrome definitions.

WHO	**NCEP:ATPIII**	**European group for the study of insulin resistance**
Diabetes or impaired fasting glycaemia or impaired glucose tolerance (hyperinsulinaemic, euglycaemic clamp-glucose uptake in lowest 25%)		Insulin resistance – hyperinsulinaemia: top 25% of fasting insulin values from non-diabetic population
Plus 2 or more of following	**3 or more of the following**	**Plus 2 or more of following**
Obesity: BMI> 30	Central obesity: waist circumference > 102cm	Central obesity: waist circumference > 94cm
Dyslipidaemia: Triglycerides> 1.7mmol/L HDL Cholesterol> 0.9mmol/L	Hypertriglyceridaemia: triglycerides > 1.7mmol/L	Dyslipidaemia: Triglycerides> 2.0mmol/L HDL Cholesterol> 1.0mmol/L
Hypertension: blood pressure> 140/90 mm Hg	Low HDL cholesterol< 1.0mmol/L	Hypertension: blood pressure> 140/90 mm Hg and/or medication
Microalbuminuria: albumin excretion > 20µg/min	Hypertension: blood pressure> 135/85 mm Hg or medication	Fasting plasma glucose>6 mmol/L
	Fasting plasma glucose>6.1 mmol/L	

Table 1.2 shows an overview of the different definitions of the metabolic syndrome taken from (Ferrannini, 1997; NECPATPIII, 2002; WHO, 2005).

1.1.3 WHO Strategy

The WHO has devised a global strategy for prevention and control of CVD. This strategy is integrated into the overall WHO chronic disease prevention and control framework for the Department of Chronic Disease and Health Promotion. The objectives of the strategy are to raise awareness of the global CVD epidemic and

to provide healthy environments for the poor and disadvantaged populations. The primary aim is to slow and reverse the trends of CVD risk factors, such as physical inactivity and unhealthy diet and therefore prevent premature deaths and avoidable disability (WHO, 2005). In order to devise an effective strategy, researchers, clinicians and policy-makers need to have a full understanding of the contribution that diet, activity, social factors, deprivation and other modifiable risk factors make to the aetiology of CVD.

1.2 Traditional view of disease risk

Until relatively recently, it was believed that the risk that an individual would develop a non-communicable disease, such as coronary heart disease CHD or type 2 diabetes, was largely determined by a persons genetic susceptibility. Recent advances in the field of genetics has led to the recognition of monogenic forms of familial hypertension and CVD (Guidi *et al.*, 2001), and new insights in research into the impact of the role single nucleotide polymorphisms (SNP) such as ApoE (Minihane *et al.*, 2007) play in the development of such diseases have provided evidence that genetic susceptibility is of high importance. Indeed a genetic predisposition coupled with an individuals modified interaction with the adult environment, encompassing lifestyle factors such as diet, physical activity levels, smoking and alcohol consumption are paramount in determining the disease risk (Langley-Evans, 2004). However, over the last decade there has been a wealth of interest in prenatal, or programmed, factors and the role they play in contributing to the onset of disease.

1.3 Fetal Programming

Evolution occurs as a consequence of adaptations to the genome that ensure the survival of species. Plants and animals alike are capable of developing in a variety of ways, developing specific characteristics in order to adapt to the environments in which they live (Bateson *et al.*, 2004). Many organisms are able to express specific adaptive responses to prevailing conditions within their environments. These responses can be short-term or intermediate changes in physiology and behaviour (Bateson *et al.*, 2004). For example the offspring of a freshwater crustacean Daphnia develop a defensive helmet that protects them from predators if the mother has been exposed to chemical traces of a predator. The protective helmet can however be a liability in a predator free environment as its construction cost reduces competitive success relative to non-helmeted individuals (Tollrian, 1999). However, it is becoming increasingly apparent that changes may be induced in the earliest stages of development as a primary response to prevailing conditions. These responses are important in the context of disease as they are likely to exert permanent effects on developing tissues. Thus early life factors can alter the phenotype that is expressed from the inherited genotype.

Preliminary research in this area began with Kermack *et al.*, (1934), who inspired the original hypothesis that early life events were linked to later health outcomes (Kermack *et al.*, 2001). However it was Rose in 1964 who sparked off current interest in the topic. Rose noted that the incidence of ischaemic heart disease was up to twice as great in individuals who had siblings that were stillborn, or that had died in early infancy (Rose, 1964). Forsdahl expanded on this concept by adding that cardiovascular mortality was of the highest incidence in areas with a high level

of infant mortality (Forsdahl, 1977). In 1979, Ellison presented data linking low birth weight and high blood pressure in 7 year olds (Ellison, 1979). Following that, Simpson *et al.*, (1981) reported a negative association between birth weight and blood pressure (Simpson, 1981). These observations were followed by a number of similar investigations (Gennser *et al.*, 1988; Ounsted, 1985; Wadsworth *et al.*, 1985). Finally Barker (1989) expanded on these original ideas to produce the "fetal origins" hypothesis (Barker, 1994). The Barker hypothesis suggested that maternal undernutrition promotes the retardation of fetal growth and that the principal markers of this are a low birth weight or disproportion (Barker, 1994). The hypothesis was based on the assumption that environmental stimuli are able to reset the developmental path of the embryo leading to restricted intrauterine growth. It has been proposed that it is this reset that predisposes an individual to non-communicable adult diseases (Moore and Davies, 2001).

Lucas (1991) was the first to try and define programming in the context of human biology and health. He described programming as the permanent response of an organism to a stimulus or insult during a critical period of development. The developing fetus is said to go through a number of critical or vulnerable, periods where organogenesis and differentiation take place. These critical periods are the stages of development when the fetus is most vulnerable to variations in nutrient or oxygen supply (Lucas, 1992). It is proposed that physiological programming occurs by two possible means. Firstly, the induction, deletion or impaired development of a somatic structure, resulting from an adverse stimulus or insult during a critical period of development, will perturb long-term function. Secondly a physiological "setting" may occur in response to an early stimulus or insult in a critical period. This causes long-term changes in function (Lucas, 1998). In simple terms, this definition

suggests that when a fetus or neonate is exposed to a suboptimum environment during a rapid phase of growth, the resulting adaptive response may become permanently fixed in place. In terms of health and well-being many years later, the effects of programming may be profound, as irreversible maladaption may result in detriment to organ function or longevity (Langley-Evans, 2004). This principle has been demonstrated by the classic Hertfordshire, Preston and Sheffield cohort studies (Section 1.4.3), where Barker and colleagues suggested that a variety of anthropometric markers at birth, including low birth weight, low ponderal index and reduced head circumference were directly related to elevated levels of coronary heart disease (CHD) risk factors and increased CHD mortality (Barker *et al.*, 1989; Hales *et al.*, 1991; Osmond *et al.*, 1993; Phipps *et al.*, 1993).

1.4 Epidemiological studies

Research into the fetal programming of disease in humans is at a relatively early stage. From a practical perspective, it is implausible to directly examine the relationship between maternal undernutrition and fetal growth, development and subsequent progression towards disease at a cellular or molecular level. It is not ethical to control maternal nutritional intake in humans (Worthington-Roberts, 2000). Human epidemiological research is therefore mainly restricted to cross-sectional retrospective studies that assess relationships between proxy markers of maternal nutrition such as birth weight and other body measurements at birth (Barker *et al.*, 1989). The criticisms of such an approach are discussed in section (1.6) and the research has so far provided insufficient conclusive data to either support or reject the fetal origins hypothesis. However, more recently prospective cohort studies have

been initiated which will examine the impact of maternal nutrition on the subsequent development of the offspring (Section 1.4.4). The following sections (1.41-1.4.4) will summarise the epidemiological evidence currently available.

1.4.1 Dutch Hunger Winter (1944-1945)

The Dutch Hunger Winter has spawned a broad body of epidemiological evidence supporting the hypothesis that prenatal nutrition is linked to an increased susceptibility to adult diseases. It occurred towards the end of the Second World War during the winter of 1944-1945 in the western parts of the Netherlands

Near the end of World War II food supplies in the Netherlands became increasingly sparse. After the D-Day landings of June 1944, allied forces broke through Nazi lines and by early September had taken back control of the majority of France, Luxembourg and Belgium. On the 14th September the allies entered the Netherlands and consequently soon liberated the southern part of the country. However their liberation efforts faltered when operation “Market Garden” (an attempt to gain control of the bridge across the Rhine at Arnhem) failed. In order to further allied liberation efforts the exiled Dutch government appealed for a railway strike, the Nazi’s retaliated by putting an embargo on all food transports to the western Netherlands. By November 1944 the embargo was partially lifted, allowing restricted food transports over water, however the unusually harsh and early Dutch winter had set in, and the canals had frozen making them impassable for barges. Food supplies within the western cities of the Netherlands soon ran out and a severe rationing scheme was put into practice. The retreating Nazi army destroyed bridges and locks in order to flood the country and halt the allied advance. These factors

combined to make the Netherlands one of the main western battlefields, resulting in the destruction of agricultural land and making transport of existing food stocks impossible. By the end of November 1944 adult rations in cities such as Amsterdam had dropped below 1000 Kcal per day and by the end of February 1945 had fallen further, to just 580 Kcal per day (Stein, 1975). The famine lasted for many months during which over 20,000 people died. During the start of the rationing period of the winter, pregnant women received extra allowances however as the famine grew more severe it became impossible to maintain these extra allowances and many pregnant women went without. Even during the most severe parts of the famine women still conceived and gave birth (Roseboom, 2001).

The Dutch Hunger Winter has provided a unique research opportunity, as there are only a few such famines in which data for daily food intake is available, After liberation (June 1945) food rations were returned to normal (~2000kcal per day) and the offspring of the Dutch Hunger Winter were not subjected to further bouts of famine. The Dutch Hunger Winter therefore provides an opportunity to research the effects of a nutritional insult on the programming of adult diseases and researchers have extensively studied hospital records in order to track individuals for assessment for a range of conditions associated with adult diseases (Ravelli, 1998, 1999; Ravelli, 1976; Roseboom, 2000, 2001).

In 1976 Ravelli and colleagues performed a cohort study of 300,000 19-year old men exposed to the Dutch Hunger famine of 1944-1945 in order to test the hypothesis that prenatal and postnatal nutrition impact upon obesity. The outcomes of the study were dependant on the time of exposure. It appeared that if the fetus was subjected to the famine in the last trimester of pregnancy, or the first months of postnatal life, the subsequent risk of obesity was reduced. However if the fetus was

exposed during the first half of pregnancy, this resulted in significantly higher obesity rates (Ravelli, 1976). Further to this Ravelli and colleagues showed the same effect in 50-year old women, but did not observe any effect on adiposity in men (Ravelli, 1999).

In 1998 Ravelli and colleagues studied glucose and insulin responses in 702 people born between Nov 1, 1943 and Feb 28, 1947 in Amsterdam for whom they had detailed birth records. They compared results of people who had been exposed to the famine at any stage of gestation, with people who were born the year before, or conceived the year after the famine. The results of this study showed that prenatal exposure to famine, particularly during late gestation was linked to decreased glucose tolerance in adulthood and that the effect of famine on fetal growth was relatively small (Ravelli, 1998). More recently Roseboom and colleagues (2000) have reported that exposure to famine was linked to an increased atherogenic lipid profile (Roseboom, 2000), and also associated with a higher than average BMI and subsequent risk of coronary heart disease (CHD) in adulthood, (Roseboom, 2001). Painter and colleagues (2005) also demonstrated that the occurance of microalbuminuria (MA) an indicator endothelial dysfunction and an important prognostic marker for kidney disease, diabetes and hypertension was increased by 5% in individuals who were exposed to the Dutch famine in mid gestation which is a critical time in determining nephron endowment in humans (Painter *et al.*, 2005). This suggests a link between fetal nutrition, nephron endowment and the subsequent development of MA and disease in later life.

1.4.2 Leningrad Siege Study

The Siege of Leningrad, also known as the Leningrad Blockade, was one of the major events of the Second World War. This event provides researchers with a unique opportunity to study the effects of more severe and prolonged nutritional programming on later health outcomes. The Leningrad siege was one of the longest sieges in modern history lasting from 8th September 1941 until 27th January 1944, during which a Nazi blockade prevented supplies from reaching the city for 872 days. At its worst, the city's inhabitants received a limited bread ration equivalent to 300 kcal per day.

In 1947 Antonov (Antonov, 1947) noted that birth weights were significantly reduced in children born during this time and since then, Stanner and colleagues have performed a number of studies on the individuals exposed to the famine *in utero*. In contrast to the results found by Ravelli *et al.*, (1998) and Roseboom *et al.*, (2000, 2001) in the Dutch Hunger Winter, there was no evidence that fetal exposure to undernutrition was linked to dyslipidaemia, glucose intolerance, hypertension or cardiovascular disease (Stanner, 1997). However an increased risk of obesity in individuals exposed to the famine was apparent (Stanner, 1997). The differing results obtained from the Dutch and Soviet cohorts may be due in part to the duration of exposure to the famine, coupled with the differing conditions experienced by the affected people in both pre- and post- famine eras.

1.4.3 Retrospective Cohort Studies

Other studies which suggest a link between birth weight and disease include the Hertfordshire, Sheffield and Preston cohort studies which considered people born

in the UK during the 1920's and 1930's. These studies suggested that low birth weight, increased ratio of placental weight to birth weight, low ponderal index, abdominal circumference and reduced head circumference were all associated with increased CHD risk factors and mortality (Barker *et al.*, 1989). Lower birth weight has also been linked to impaired glucose tolerance, non-insulin dependent diabetes and high blood pressure (Hales and Barker, 1992; Hales *et al.*, 1991). Another study which inferred a link between birth measurements and type II diabetes and hypertension was the Helsinki cohort, in which 13 517 men and women born at Helsinki hospital in 1924-1944 were observed to have a higher risk of type II diabetes and hypertension if their birth weight was less than 3kg and pre-pubertal BMI was in the highest quartile, compared to those whose birth weight was more than 4kg and whose BMI was in the lowest quartile (Barker *et al.*, 2002a). The relationship was observed to be stronger when CHD risk was assessed relative to ponderal index (birth weight/birth length) in men, and to length at birth, in women. Disease risk was also related to rate of growth in childhood.

When analyzing associations in epidemiological studies it is important to appreciate their limitations. Clearly data from the Dutch and Leningrad studies conflict and it is important to understand that these studies are retrospective and that although the general food intake of the participants was known, it is unrealistic to assume that these values are absolute and consistent. Also the studies of the Dutch Hunger Winter assessed offspring according to different prenatal exposure time points whereas the Leningrad study simply assessed those who were, or were not, exposed (Stanner and Yudkin, 2001). This point is particularly relevant as a wide range of animal studies have shown that nutritional restriction at specific time points in fetal development have differing effects (Langley-Evans, 1999a; Langley-Evans *et*

al., 1996b). It has also been noted that conception rates, especially in the poorer social classes, declined during both the Dutch Hunger Winter and the Leningrad siege. It has therefore been hypothesized that the observed programming could be due to social class of the parents rather than nutrition (Stein *et al.*, 2004). It is also important to note that the impact of wartime stress can not be dissected out from the impact of undernutrition.

1.4.4 Prospective cohort studies

Currently underway in both the United States and Southampton, UK are several ground-breaking prospective longitudinal cohort studies including "Project Viva", the "Avon longitudinal study of pregnancy and childhood" and the "Southampton Women's Study" (Inskip *et al.*, 2009; Lawlor *et al*., 2005; Ong *et al*., 2000). These studies aim to improve the health of the mother and child by looking at the effects of diet and other environmental factors during pregnancy.

Project Viva is an ongoing study funded by the National Institutes of Health (NIH), which began enrolling pregnant women in April 1999, and to date has enrolled a total of 2670 subjects. The study is in its seventh year and has so far yielded some interesting results. Gillman and colleagues (2004) analysed 4091 Dinamap blood pressure measurements from a total of 936 6-month old infants. Second trimester food frequency questionnaires of the mothers were also analysed and it was determined that every 500-mg increment of maternal calcium supplementation during the second trimester of pregnancy resulted in a 3.0 mm Hg decrease in systolic blood pressure in the children at 6-months of age (Gillman, 2004). Similarly Lawlor and colleagues (2005) recently performed a prospective

cohort study that examined the association between birth weight and cardiovascular disease end points in 10,803 singleton individuals born within the 1950s, which was a time when environmental circumstances, (indexed by low infant mortality rates), were relatively advantageous for infants. This study suggested that birth weight is inversely associated with CHD and stroke (Lawlor *et al*., 2005). A further prospective study was performed by Ong and colleagues (2000) who analysed the association between postnatal catch-up growth and obesity in childhood in 848 full term singletons from a 10% random sample of the Avon longitudinal study of pregnancy and childhood and found that Children who demonstrated catch-up growth by 2 years of age exhibited more central adiposity at 5 years of age than other children (Ong *et al*., 2000).

1.5 Hypotheses of Fetal Programming

1.5.1 The Thrifty Genotype Hypothesis

The fundamental origins of non-communicable disease are clearly multi-factorial. To date opinion has favoured the role of genetic susceptibility, where a specific genotype, that is apparent in different ethnic groups, has the potential to infer a predisposition to disease (Groop and Orho-Melander, 2001; Neel, 1962). The thrifty genotype hypothesis was derived when Neel and colleagues observed that certain ethnic groups had a tendency to develop type II (non-insulin dependant) diabetes mellitus with an ever increasing prevalence, Neel (1962) postulated that evolutionary pressures from consistent famine had created a new human genotype. This new genotype had increased chances of survival at times of severe nutritional sparsity, (Neel, 1962). However in times of abundance these genes predispose their

carriers to diseases caused by excess nutritional intake, such as obesity, due to the genes having evolved to maximize metabolic efficiency, lipid storage and food searching behaviour (Neel, 1962). The thrifty genotype hypothesis assumes that several thrifty gene varients act simultaneously, inducing altered glucose and fat metabolism via changes to organ function. The hypothesis assumes that disease is directly due to gene-environment interactions and to date the list of thrifty candidate genes thought to be involved in the origins of disease is extensive. Peroxisome proliferator activated receptors and ß-adrenergic receptors, represent a few examples from a long list (Groop, 2000).

1.5.2 The Thrifty Phenotype Hypothesis

Following the emergence of the "thrifty genotype" hypothesis Hales and Barker (1992) proposed an alternative "thrifty phenotype" hypothesis. This hypothesis incorporated understanding of the aetiology of non-communicable diseases such as type II diabetes. Hales and Barker (1992) proposed that such diseases are a consequence of environmental conditions, such as poor fetal nutrition, that are central to disease susceptibility and that genetics contributes only mildly (Hales, 2001). The biological basis of the hypothesis is that fetal undernutrition causes "brain-sparing", the situation where a fetus subjected to undernutrition diverts blood flow to the brain at the expense of the other organs. The other organs adapt to an environment of sparse nutrition and this can have a major effect on their resulting structure and later function (Hales and Barker, 1992). The thrifty phenotype also represents a way in which a pregnant female can modify the development of her unborn fetus so that it will be prepared for survival in an environment in which

resources are likely to be scarce. The acquisition of a thrifty metabolism will provide a survival advantage if environmental conditions remain the same once the fetus has been born, consequently increasing the probability the fetus will reproduce (Hales, 2001). Similar to the thrifty genotype hypothesis the thrifty phenotype is likely to be a disadvantage and the individual may suffer disease due to metabolic efficiency in an environment where energy is consumed in excess (Langley-Evans, 2004; Leon, 2004).

The thrifty phenotype, with enhanced metabolism, can be observed in many different populations throughout the world which have undergone the transition from poverty to relative affluence (Langley-Evans, 2004). For example, Cohen *et al.*, (1988) noted that a group of Jewish migrants known as the Falashas who moved from Ethiopia to Israel, and therefore from an area of common famine to a westernised nation, exhibited a rise from 0.5% to 18% prevalence of non-insulin-dependant diabetes within 5 years. This was twice as high as the rest of the Israeli population and 30 times greater than the prevalence among Ethiopians living in Ethiopia (Cohen, 1988). This concept is particularly relevant for the next few decades, as there is expected to be an explosion of the prevalence of obesity, type 2 diabetes and cardiovascular disease in countries such as India, China and South Africa as they are making the economic and nutritional transformation to become more westernised, after many generations that would select for thrift (Robinson, 2001).

More recently the thrifty phenotype hypothesis has been adapted and now encompasses other factors such as obesity, aging and physical activity which are believed to be critically influential on the outcome of disease (Hales, 2001). Prentice and Moore (2005) suggested that a combination of both the thrifty phenotype and

thrifty genotype influence the health of populations and that thrift has increased certain populations susceptibility to non-communicable diseases (Prentice, 2005). In essence all of these previous ideas contribute to the newly emerging concept of a "lifespan" model of disease where gene-environment interactions occur throughout life (Kuh, 2003). The response of the individual to the environmental challenges at any stage of life will essentially reflect the cumulative influence of responses to such cues at earlier stages of life.

1.5.3 Predictive adaptive response hypothesis

The thrifty phenotype hypothesis is consistent with the "predictive adaptive" response theory of Gluckman and Hanson (2004), which proposes that prenatal stimuli cause the fetus to predict the future postnatal environment, and adapt accordingly for life outside the womb (Gluckman and Hanson, 2004). These adaptations may aid long-term survival, adaptations ensures survival through natural selection then the adaption is likely to be passed across generations. However it is when a mismatch between pre- and postnatal environments occurs that the adaptation is likely to be unsuitable and lead to disease (Gluckman and Hanson, 2004).

1.5.4 The Fetal insulin hypothesis

As an alternative to the fetal programming hypothesis Hattersley and colleagues (1998) proposed the fetal insulin hypothesis for the association of low birth weight and type II diabetes and insulin resistance. In essence they suggested that insulin regulates fetal growth and that subsequent birth weight is directly

determined by fetal insulin secretion and insulin action, both of which are regulated by fetal genotype (Hattersley, 1998). Hattersley and co-workers developed this hypothesis after analysing birth weight and the presence or absence of glucokinase mutations in the fetus and mother. They investigated 58 children from 23 nuclear families from 10 extended glucokinase pedigrees. In the 58 offspring, birth weight was higher in the presence of a maternal mutation and lower in the presence of a fetal mutation. However when both the mother and the fetus had the mutation, birth weight was not significantly different from when neither mother nor the fetus had the mutation. Hence a significant difference in birth weight was only seen when both the fetus and mother were discordant for the glucokinase mutation (Hattersley, 1998). As glucokinase will clearly influence glucose homeostasis and carry a diabetes risk, the presence of these mutations confounds relationships between birthweight and diabetes risk. These observations have demonstrated that there are important implications for the study of genetics in adult diseases. Although glucokinase mutations are relatively rare within the population and not numerically important in type II diabetes (Hattersley, 1998), it is clear that genes have the potential to influence birth weight. Although environmental influences such as undernutrition and maternal smoking clearly have some impact, the study of the genetics of low birth weight may help to remove some of the uncertainties surrounding diabetes and hypertension.

1.5.5 Summary of disease factors

It is clear that the aetiology of disease is multi-factorial. Disease risk will stem from interactions of all factors genotypic and phenotypic and these factors have

the potential to influence well-being at all stages of the life course. It is also important to recognise the complexity of the disease process and accept that our understanding of the process is limited. For example it is now apparent that the Falashas carry a haplotype that predisposes the population to β-cell destruction and therefore type 1 diabetes (Zung *et al.*, 2004). This further complicates interpretation of data which supports the thrifty phenotype hypothesis. Data from both human and animal studies continue to provide essential insights into the process of disease.

1.6 Criticisms of the programming hypothesis.

Research into the impact of fetal programming on humans is at a relatively early stage and the full list of factors that may act as stimuli are not known. However, work done by Barker and colleagues has proposed maternal nutrition to be of prime importance in determining the long-term risk of disease (Barker *et al.*, 1993). Barker suggested that maternal undernutrition promotes retardation of fetal growth, which is observable as low birth weight or disproportion (Barker, 1994). This is now known as the Barker hypothesis. However, unlike many other species, the growth of the human fetus does not seem to be very susceptible to alterations in maternal diet, and human growth rates appear to be largely determined genetically, although they may be constrained by lower socio-economic status (partly diet, smoking and maternal size) (Campbell, 1991). Some studies show some impact of undernutrition on birth weight, but these are extreme cases where a severe famine has taken place, and even then the effects on birth weight are marginal (Prentice, 1987).

Women in the United Kingdom have been the subject of many studies on the relationship between diet and birth weight and there is very little evidence that variation in maternal diet effects birth weight (Doyle, 1992; Mathews *et al.*, 1999). There is also very little evidence that maternal diet alters body proportions at birth (Langley-Evans and Langley-Evans, 2003). The Barker hypothesis has also been strongly challenged by Bartley *et al.,* (1994) who demonstrated that the cause of low birth weight was more likely to be related to socio-economic status, and that these socio-economic factors continued to influence the individual into adult life (Bartley *et al.*, 1994). Kramer and Joseph, (1996) have also pointed out inconsistencies within Barker's work and studies by Matthes *et al.,* (1994) have found no link between characteristics at birth and cardiovascular risk (Kramer and Joseph, 1996; Matthes *et al.*, 1994).

Major criticism of the Barker hypothesis also comes from Huxley *et al.*, (2002) who performed a meta-analysis of over 100 studies that had previously reported a regression coefficient of systolic blood pressure in relation to birth weight. Huxley *et al.,* found that the inverse association between birth weight and blood pressure may simply have been due to random error and an inappropriate adjustment for confounding factors such as current weight (Huxley *et al.*, 2002).

1.7 Animal models as proof of the programming principle

Although there seems to be much controversy surrounding the idea that maternal nutrition and prenatal undernutrition programmes human disease, since the 1960's there has been a broad body of epidemiological evidence to add support to the claim. However, most of the more robust evidence comes from animal models,

especially studies of the pregnant rat and the sheep. Such studies show that exposing the developing fetus to maternal food restriction or restriction of specific nutrients results in changes to normal physiology and the initiation of disease processes (Bertram and Hanson, 2001; Langley-Evans, 2004; Langley-Evans, 2001)

Animal studies have been extensively used to explore the programming principle and a wide range of programmed endpoints have been identified. These include obesity, (Breier *et al.*, 2001; Jones, 1983), insulin resistance, (Simmons *et al.*, 2001), impaired glucose homeostasis (Taylor *et al.*, 2005), hypertension (Dunn *et al.*, 2001; Khan *et al.*, 2003; Langley and Jackson, 1994), susceptibility to oxidative damage (Jennings *et al.*, 2000), adiposity (Guo and Jen, 1995) and impaired immunity (Chisari *et al.*, 2001). These endpoints are observed after restriction of food intake (Woodall *et al.*, 1996a; Woodall *et al.*, 1996b) or of specific nutrients such as iron (Crowe *et al.*, 1995; Gambling *et al.*, 2003), calcium (Bergel and Belizan, 2002) sodium (Battista *et al.*, 2002), zinc (Beach, 1982) and protein (Langley and Jackson, 1994) or via overnutrition by feeding a fat-rich diet (Khan *et al.*, 2004; Khan *et al.*, 2003; Taylor *et al.*, 2005). Programming by nutritional manipulation has been observed in many different species such as sheep, (Gopalakrishan, 2004) guinea pigs, (Kind, 1999) rats, (Langley-Evans, 2001) and mice (Ozanne *et al.*, 2004). This review will concentrate on the restriction of protein in rodents, which is one of the most studied nutrients in fetal programming.

1.8 The maternal low protein diet model

Out of the many different nutrients protein is particularly relevant to global nutrition because in humans protein intakes vary globally and even regionally.

Intakes also vary in relation to socio-economic class. Data extracted from 2004 Food and Agriculture Organisation of the United Nations (FAO) indicate that 65% of the world population are likely to consume protein at less than the UK Reference Nutrient Intake (51g/day), and therefore are at risk of low protein intake during pregnancy (FAO, 2004). It is important to note that this information is derived from food balance sheets which only determine the available protein (i.e. produced through agriculture or imported) per head of population. Therefore actual consumption will generally be lower and highly variable. Further to this in 2002 a study of trimester 1 and 3 intakes of 300 British women was completed (Langley-Evans, 2004). This study found that 6-8% of the women had first or third trimester protein intakes below the UK Reference Nutrient Intake and that 55% of these individuals were from lower socio-economic groups (Langley-Evans, 2004).

Low protein diets were first used to promote fetal growth retardation in rats after Zeman and Stanbrough, (1969) found that feeding pregnant rats diets containing 40-60 g protein/kg, produced severe reductions in offspring birth weights (Zeman and Stanbrough, 1969). Many early studies used diets that contained 4-5% protein with controls containing up to 24% protein to examine this effect. This is not likely to be physiologically relevant as the pregnant rat requires 12% protein (Clarke *et al.*, 1977). Langley and Jackson (1994) reported that feeding a 60, 90 or 120g protein/kg diet to pregnant rats caused increased blood pressure in their offspring, compared to that of control rats, despite all rats being transferred to the same standard diet at littering (Langley and Jackson, 1994).

Langley-Evans and colleagues have since extensively characterised this low protein diet model of programming in the rat. The control diet for these studies contains 18% casein and various levels of protein restriction have been applied (12-

6%) (Langley-Evans, 2004). Most work has concentrated on a 9% casein diet (Langley-Evans, 2001), as this is the protein requirement for a non-pregnant rat and therefore represents only a mild restriction (Langley-Evans, 2004). Langley-Evans and colleagues matched the energy of the low protein diet with the control diet by increasing carbohydrate content in proportion to the protein restriction (Langley-Evans, 2004). The programming effects of this diet have been monitored comprehensively and are explained below.

Rats that are fed a low protein diet throughout pregnancy (9% casein) have fetuses that are larger than those of control specimens from day 14 through to day 20 of gestation, and this effect is amplified if low protein feeding continues for only the first 7 days of gestation (Langley-Evans *et al.*, 1996d; Langley-Evans and Nwagwu, 1998). Placental size is related to fetal growth and feeding of a low protein diet produces an enlarged placenta by day 20 of gestation (Langley-Evans, 2001). During rat gestation the rat fetus doubles in weight over the final 2 days (day 21 and 22). The feeding of a low protein diet retards this major surge in growth and by full- term the offspring tend to be of low- to- normal birth weight (Langley-Evans *et al.*, 1996d). This late gestation growth retardation appears to primarily affect the development of the truncal organs, such as the liver and the lungs. Body length is also reduced but the brain remains unaffected (Langley-Evans *et al.*, 1996d). Adult body composition does not appear to differ between low protein exposed rats and control rats, however there is some evidence that low protein-exposed male rats deposit more gonadal fat with aging (Bellinger *et al.*, 2004; Bellinger *et al.*, 2006). Roach *et al.*, (1999) also found that animals exposed to a low protein diet had abnormalities in their bone morphology (Roach *et al.*, 1999). Hales and colleagues have also noted that fasting plasma glucose and insulin levels are lower in the

offspring of rats fed a MLP diet *in utero* (Hales *et al.*, 1996). This reduction is associated with improved insulin sensitivity in early adulthood, however by 15 months of age, glucose tolerance in MLP offspring is noted to significantly decline compared to controls and MLP exposure is associated with hypoinsulinaemia in female animals and hyperinsulinaemia in male animals (Hales *et al.*, 1996).

The major focus of research into the effects of MLP diet on fetal development, is the effect the low protein diet has on blood pressure. By 4 weeks of age rats consistently exhibit raised systolic blood pressure if exposed to maternal protein restriction *in utero* (Langley-Evans, 2001). The increase in blood pressure varies between 7 and 30 mmHg (Langley-Evans *et al.*, 1994; Langley and Jackson, 1994) and appears to be permanent, with blood pressure remaining elevated in adult life (Langley-Evans and Jackson, 1995). No differences between males and females have been found and generally the elevated blood pressure is found to go hand in hand with a reduced heart rate (Nwagwu *et al.*, 2000). Similar experiments have also been performed in mice with the same outcome (Dunn *et al.*, 2001). The timing of low protein feeding does not appear to be critical in programming raised blood pressure. Feeding of a low protein diet for single weeks in pregnancy produces raised blood pressure. However, low protein feeding over the final week of gestation was found to be associated with greater elevation than earlier insults (Langley-Evans *et al.*, 1996b), but the greatest increase was found when feeding a low protein diet throughout gestation (Langley-Evans, 2004).

In contrast to the low protein diet used by Langley-Evans and colleagues, the low protein diet (Hope farms diet) used by Hales *et al.*, (1996) for investigations in to glucose metabolism and insulin resistance does not programme blood pressure changes (Langley-Evans, 2000). The Langley-Evans low protein diet differs in

several ways, it contains extra methionine (to avoid sulphur deficiency associated with a casein based diet), fat and carbohydrate are provided as maize oil and sucrose-starch respectively. The Hope farms diet provides less than half the amount of fat (soyabean oil) and provides carbohydrate mostly in the form of glucose (Langley-Evans, 2000). It is therefore very important that the composition of diets used in all investigations are fully published, so that work can be compared and contrasted. Similarly the low protein diet utilised by John Betram's research group also fails to program blood pressure (Hoppe et al., 2007a). However, it must be noted that although the composition of the Hoppe diet differs very little from the diet utilised by Langley-Evans and colleagues, the Hoppe diet was administered both pre- and postnatally and therefore a direct comparison can not be made.

The method utilised to monitor blood pressure must also be carefully described as there is much controversy over which is the most accurate method to use in order to monitor blood pressure. Opinion within the scientific community favours the use of radio telemetry systems. However, these are both invasive and expensive. Indirect tail-cuff methods allow the analyses of blood pressure without the use of invasive procedures which may themselves alter blood pressure. Modern systems allow measurements to be taken without the use of heating, therefore preventing heat-stress of the animals (Kubota *et al.*, 2006). Analysis of the two different methodologies have concluded that the tail-cuff method is a sensitive and accurate technique for determining blood pressure in conscious rats (Ibrahim *et al.*, 2006) and it has demonstrated that similar measurements are obtain by both the radio telemetry and the unheated tail-cuff methods (Kubota *et al.*, 2006). Work within our research group has previously utilised the tail-cuff method of monitoring blood pressure. The programming effects of the low protein diet upon blood pressure have

been verified by other research groups using the radio telemetry method (Woods *et al.*, 2004).

It has been suggested that low protein diet of Langley-Evans and colleagues (1994) programmes blood pressure by altering the structure of the vasculature and the kidney. Langley-Evans *et al.*, (1999a; 2002) found that rats exposed to a low protein diet had a reduced number of nephrons in the kidney at the time of birth, similarly Hoppe and colleagues (2007a; 2007b) have also noted a reduced nephron complement in both rats and mice following maternal protein restriction utilising a different low protein diet (Hoppe et al., 2007a; Hoppe *et al*., 2007b). Mid-to-late gestation is thought to be a critical period for the development of the kidney (Langley-Evans *et al.*, 2003; Langley-Evans *et al.*, 1996b). Mackenzie and Brenner, (1995) argued that in order to maintain the glomerular filtration rate (GFR), local blood pressure inside the glomerulus is increased by the renin-angiotensin system (RAS), maintaining glomerular perfusion (Mackenzie, 1995) which impacts on systemic blood pressure. Martyn and Greenwald (2001) have argued that programming of the vascular structure contributes to hypertension (Martyn and Greenwald, 2001), and it has been demonstrated that low protein feeding in the rat produces noticeable changes within the ascending aorta such as thinner-walled vessels and differing elastin and collagen deposition from as early as 4 weeks' postnatal age (Langley-Evans, 2004).

One of the major problems with using rodent models for cardiovascular disease is that rats are resistant to atherosclerosis and CHD. Therefore there have been very few rodent studies that examine CHD markers other than blood pressure, (Langley-Evans, 2004). A recent study by Yates and colleagues (2008) however has circumvented this problem by using ApoE*3-Leiden transgenic mice. ApoE*3-

Leiden mice carry a mutation of human ApoE*3 rendering them prone to atherosclerosis when fed a diet rich in cholesterol. Yates and colleagues found that fetal exposure to a low protein diet *in utero* resulted in dyslipidaemia, elevated total plasma cholesterol and triglyceride concentrations and the development of more severe atherosclerotic lesions within the aortic arch compared to control animals when fed an atherogenic postnatal diet (Yates *et al.*, 2008). This model for the first time effectively demonstrates the interaction of prenatal undernutrition with a postnatal atherogenic diet increases the extent of atherosclerotic disease. Subsequent studies and further investigation is needed in this area.

In conclusion, there is a wealth of experimental evidence that suggests that fetal exposure to undernutrition (low protein diet) may be a factor capable of programming cardiovascular disease. The mechanisms behind programming are not fully understood, and more work must be done in order to understand completely the nature of the risk associated with programming.

1.9 Mechanisms of programming

Research into the mechanisms behind the programming phenomenon is at a very early stage and more investigation into the topic is needed. The exact mechanism behind programming is not fully understood. An understanding of the molecular machinery behind programming is essential in order to curb the rising trend in non-communicable adult disease and in establishing therapeutic targets. The key potential mechanisms thought to be involved in the nutritional programming of disease are discussed below.

1.9.1 Glucocorticoid hypothesis

Glucocorticoids are steroid hormones that are essential for normal physical development of the fetus *in utero*. Exposure to glucocorticoids promotes the maturation of fetal tissue *in utero* preparing the fetus for life outside the womb (Edwards *et al.*, 1993). In the lungs, glucocorticoids initiate the thinning of the alveoli and production of surfactant (Ward, 1994). Glucocorticoids are routinely given to women as they enter preterm labour in order to facilitate pulmonary development. As well as their role in development, glucocorticoids are also important mediators of stress responses and metabolic functions such as the stimulation of fat breakdown in adipose tissue and are involved in many signalling pathways (Cato *et al.*, 2002; Rhen and Cidlowski, 2005). The binding of glucocorticoids to their receptors enables transcription of gene targets (Newton, 2000). The development and maturation of the hypothalamic-pituitary adrenal (HPA) axis is important in fetal development, occurring late in gestation. It is especially sensitive to glucocorticoids (Edwards and McMillen, 2002). As a consequence of this the placenta acts as a critical barrier, actively preventing glucocorticoids passing from the mother to the fetus. Consequently there is a large concentration gradient of active glucocorticoids between maternal and fetal circulation, which can be as high as 1000:1 (Edwards *et al.*, 1993). The gradient is maintained by the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD 2), which, in the placenta, catalyses the conversion of active glucocorticoids to inactive metabolites (Drake and Seckl, 2004; Edwards *et al.*, 1996). This prevents the fetal tissues being overwhelmed by active glucocorticoids that would perturb HPA axis development, ensuring that the HPA axis is allowed to develop independently of maternal influences.

The programming effects of glucocorticoids upon the development of chronic disease states are well established. Animal studies in both sheep (Tangalakis *et al.*, 1992) and rodents (Nyirenda *et al.*, 1998) suggest that fetal exposure to synthetic glucocorticoids such as dexamethasone and betamethasone, which are poor substrates for 11β-HSD 2, have programming effects upon the development of hypertension and renal disease (Benediktsson *et al.*, 1993; Celsi *et al.*, 1998; Dodic *et al.*, 2002). Indeed Benediktsson and colleagues found that the administration of dexamethasone during pregnancy resulted in offspring with lower birth weights and higher blood pressure than control animals (Benediktsson *et al.*, 1993).

Due to the similarities between phenotypes exhibited by animals exposed glucocorticoids and the nutritional insults during gestation, it has been proposed that undernutrition during gestation may act as a stressor, altering the glucocorticoid gradient between the mother and fetus (Langley-Evans, 2008). Indeed the feeding of a low protein diet during gestation has been shown to reduce the placental activity and expression of 11β-HSD 2 (Langley-Evans *et al.*, 1996c) resulting in over-exposure of the fetus to maternal glucocorticoids. It has been proposed that it is this over-exposure that initiates tissue remodelling resulting in the programmed phenotype associated with the low protein diet (Langley-Evans, 1997). Studies in rats support this hypothesis as when fed a low protein diet during gestation combined with a treatment of metyrapone, a drug which blocks glucocorticoid synthesis in early to mid gestation, offspring fail to develop the programmed phenotype of hypertension and renal insufficiency associated with the low protein diet (McMullen and Langley-Evans, 2005).

Even with the abundance of work within the field of glucocorticoids the mechanisms through which over-exposure promotes programming are still unknown.

It is established that glucocorticoids promote tissue maturation and this takes the form of differentiation rather than proliferation. Current thinking is that during development this may result in tissue remodelling, leading to the production of fewer functional units within tissues (Langley-Evans, 2008). This is a process which has previously been noted in the kidney of sheep (Dodic *et al.*, 2002) and rodents (Vehaskari *et al.*, 2001).

1.9.2 Tissue remodelling

Normal tissue development involves both proliferation and differentiation. All tissues and organs develop from progenitor (unipotent) cells in the embryo which expand down the appropriate lineage during organogenesis. The timing of organogenesis differs between tissues, for example the kidney develops late in gestation, whereas the heart is formed early in development (Langley-Evans, 2004). As previously stated organogenesis takes place as two specific stages proliferation and differentiation. Current thinking is that a nutritional insult during a critical period of development such as organogenesis could interfere with either process (Brameld *et al.*, 1998). This would result in either a reduced organ size, or a reduction in functional units depending upon which phase of organogenesis was impeded. Several animals models of nutritional programming exhibit evidence of tissue remodelling. For example, rats exposed to a low protein diet *in utero* exhibit a 30-40% reduction in nephron complement when on gross examination kidney sizes are similar (Langley-Evans *et al.*, 1999a; Vehaskari *et al.*, 2001). Studies in sheep have yielded similar results (Gopalakrishnan *et al.*, 2005). Remodelling of the brain has also been noted. The feeding of a low protein diet has been demonstrated to remodel neuronal

density (Plagemann *et al.*, 2000) and vasculature (Bennis-Taleb *et al.*, 1999). It is clear that tissue remodelling could be of paramount importance as a cause of disease following fetal undernutrition, however it is still unclear what underlying processes are involved in driving the remodelling of tissues.

1.9.3 Epigenetic mechanisms

The expression of key genes during development is thought to be regulated by a number of epigenetic mechanisms. Whereby changes in gene expression are mediated by changes in transcription by modulating access of the transcriptional machinery to the DNA strands (Langley-Evans, 2008). DNA methylation and histone acetylation, which control the super-coiling of DNA around the histone proteins within each chromosome, can decrease and increase transcription of genes. The role of acetylation in promoting transcription and methylation in promoting silencing, are thought to be of prime importance in the disease process (Bird, 2002). This section of the current literature will concentrate on the best characterised epigenetic mechanisms, DNA methylation and histone modification.

1.9.3.1 History of epigenetics

The term epigenetic was coin by developmental biologist Conrad Waddington (Van Speybroeck, 2002; Waddington, 1940) in the early 1940s to define the way in which genes and the environment can influence the mature phenotype. Waddingtons work stemmed from work on *Drosophila melanogaster*, showing that alterations in wing vein patterns could be induced by heat-shock treatment of the pupae. Breeding of the *Drosophila* that were particularly susceptible to the heat

shock treatment resulted in *Drosophila* that exhibited the phenotype without the environmental stimulus. This was termed genetic assimilation (Waddington, 1957) and essentially showed that environmental stimuli may interact with the genome during plastic stages of development, conveying phenotypical effects that may be heritable (West-Eberhard, 2003).

Further to this, in 1975 Holliday and Pugh proposed that covalent chemical DNA modifications at cytosine-guanine dinucleotides such as DNA methylation were the mechanism behind Waddingtons hypothesis (Holliday and Pugh, 1975). The discovery that X inactivation and genomic imprinting are also regulated by epigenetic mechanisms has highlighted the importance of epigenetic mechanisms within gene regulation (Monk, 1988; Willard *et al.*, 1993).

1.9.3.2 DNA Methylation

DNA methylation (Figure 1.3) is the process by which cytosines are methylated (addition of a methyl group) after DNA replication and is the best characterised form of epigenetic marking. The methylation of DNA protects it from endonuclease degradation and plays a critical role in regulating gene expression (Waterland and Michels, 2007). The structure of the genome is established during pre-implantation, in which specific DNA methylation patterns are established. Gene expression is altered by the methylation of cytosine residues within CpG islands in the promoter regions of genes (Waterland and Michels, 2007). An increase in CpG methylation is associated with a decrease in the level of expression, and so is associated with a restriction of transcriptional potential and silencing of the gene (Jaenisch and Bird, 2003). DNA methylation is thought to work with other chromatin

modifications to heritably maintain some areas of the genome in a transcriptionally silent state (Bird, 2002).

As methyl donors and cofactors necessary for the synthesis of S-adenosylmethionine, a prerequisite for DNA methylation, are derived from the diet (Van den Veyver, 2002), it has been hypothesized that early nutrition may influence adult diseases through modification of DNA methylation (Waterland and Garza, 1999). Interest in DNA methylation and its role within the field of nutritional programming was stimulated when Waterland and Jirtle (2003) tested the hypothesis that the methylation status of specific transposable element insertion sites (loci) are able to be epigenetically programmed by early availability of methyl donors. Waterland and Jirtle (2003) showed that dietary supplementation of the diets of yellow agouti (Avy) mice with folic acid, choline, vitamin B12 and betaine, altered the coat-colour phenotype of the heterozygous offspring via increased CpG methylation at the Avy locus (Waterland and Jirtle, 2003). This effectively demonstrated that nutritional factors during fetal development can modify gene expression. Further to this Sinclair and colleagues (2007) showed differential methylation of genes in fetal livers by feeding sheep a diet deficient in the methyl donors, folic acid, vitamin B12 and methionine for 8 weeks prior to conception and for the first 6 days of pregnancy. This resulted in male offspring exhibiting a phenotype of elevated blood pressure and insulin resistance. Analysis by Restriction landmark genome scanning (RLGS) revealed that 4% of the CpG islands within the fetal liver were differentially methylated and the majority of these differentially methylated CpG islands were hypomethylated (Sinclair *et al.*, 2007).

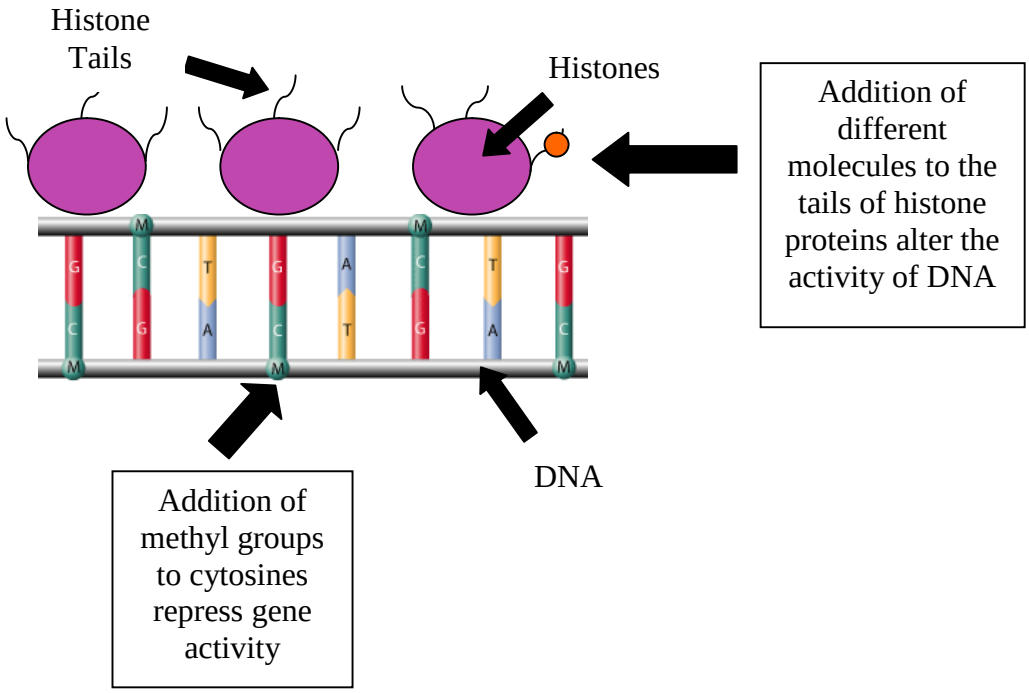
Recent research by Lillycrop and colleagues (2005) has demonstrated that the feeding of a low protein diet during rat gestation results in adult offspring over-

expressing both peroxisome proliferator activated receptor α (PPARα) and glucocorticoid receptor (GR) (Lillycrop *et al.,* 2005). The results from the studies by Lillycrop *et al.,* are comparable with the results of Sinclair and colleagues which suggest that nutritional insults such as the low protein diet lead to hypomethylation of DNA and therefore result in the overexpression of key genes in development (Lillycrop *et al.,* 2005; Lillycrop *et al.,* 2008; Lillycrop *et al.,* 2007).

The reason why nutrition *in utero* and, specifically the low protein diet, elicits these effects upon DNA methylation is unknown. Current thinking is that the expression of DNA methyltransferase 1 (DNMT-1) an enzyme involved in the establishment and regulation of tissue-specific patterns of methylated cytosines is down-regulated (Lillycrop *et al.,* 2007). The availability of methyl donors such as methionine and folic acid may also be of prime importance, as demonstrated by the work of Sinclair *et al.,* (2007). Supplementation of the low protein diet with folate during rat pregnancy prevents the establisment of the physiological phenotypes observed with the low protein diet. It has also been noted to normalize DNA methylation patterns (Lillycrop *et al.,* 2007; Torrens *et al.,* 2006). Further to this Rees *et al.,* (1999) found that the feeding of a low protein diet reduced the threonine concentration in fetal serum without affecting any other amino acid. It is thought that threonine may be used in the generation of glycine, which is required in many metabolic pathways during gestation. It is therefore thought to be a limiting nutrient especially when protein intakes are low. The hypertensive effect of a low protein diet can be reversed if the diet is supplemented with glycine (Jackson *et al.,* 2002). Rees *et al.,* (1999) proposed that glycine was important because it was involved in the metabolism of methionine and homocysteine (Rees *et al.,* 1999). An absence of

glycine would cause an accumulation of homocysteine, having implications for DNA methylation and possibly impacting on programming.

Figure 1.3 Overview of epigenetic mechanisms.



1.9.3.3 Histone modification

It is important to note that DNA methylation is not the only contributor to epigenetic regulation of gene expression. Chromatin structure, the packing of genomic DNA through association with proteins (Van Holde, 1998) is also of importance (Figure 1.3). Indeed there is emerging evidence that both histone modifications and DNA methylation are intrinsically linked (Cheung and Lau, 2005). The organisation of chromatin has the ability to restrict the access of nuclear factors to DNA and recent evidence has suggested that modification of histone proteins via acetylation, phosphorylation, methylation and ubiquitination essentially alters chromatin conformation and may play a direct role in the regulation of gene

expression (Felsenfeld and Groudine, 2003). Indeed evidence from the field of fetal programming (Lillycrop *et al.*, 2007) demonstrated that hypomethylation and increased expression of the GR110 promoter are associated with reduced DNMT-1 expression and altered histone modifications of the GR promoter, in the liver of offspring from rats fed a protein restricted diet *in utero*. A review of the role histone modifications play within the field of genetics/epigenetics is beyond the scope of this thesis. For a comprehensive review of histone modifications please see (Berger, 2002; Iizuka and Smith, 2003; Zhang, 2003).

1.9.4 Epigenetic drift

Epigenetic drift is a process that promotes either DNA hypo- or hyper-methylation with advancing age. Currently, research within the area of epigenetic drift and the emergence of age-related phenotypes that develop with age is at a very early stage. It is suggested that epigenetic drift occurs due to a decline in expression of DNMT-1, which leads to a demethylation of the whole genome. Specific tissues may up-regulate DNMT-3b as a result of this de-novo demethylation, causing hypermethylation in certain gene promoters (Casillas *et al.*, 2003; Fraga and Esteller, 2007). Research into the role epigenetic drift plays within disease development is at a very early stage but it is clear that epigenetic drift can have severe consequences for the process of disease through the alteration of gene expression. This mechanism has been associated with the development of cancers (Casillas *et al.*, 2003) and Alzheimer's disease (Wang *et al.*, 2008). Previous studies have indicated that gene expression changes throughout the life course, for example in the rat model of protein restriction the expression of genes involved in fatty acid synthesis such as

SREBP-1c were up-regulated with aging, whereas genes associated with fatty acid oxidation such as PPAR-α were down regulated with aging. Both these shifts in expression lead towards progression of the metabolic syndrome and the development of disease (Erhuma *et al.*, 2007a).

1.10 Transgenerational Programming

Epidemiological studies have indicated that suboptimal nutrition during fetal development may permanently programme offspring's susceptibility to chronic diseases such as CHD and diabetes. However these effects of fetal programming may not be limited to the first generation, as there is newly emerging evidence that transgenerational effects may occur, whereby the consequences of deficits in maternal nutrition are subsequently passed on to the grandchildren (James, 2002). Pembrey (1996) suggested an intergenerational feed-forward control loop existed, which linked the health and growth of an individual with the nutrition of their grandparents (Pembrey, 1996).

1.10.1 Transgenerational programming in humans

Human studies in the field of transgenerational programming are very limited, as it is unplausible and unethical to directly examine the relationship between undernutrition *in utero* and the progression towards disease, time is also a limiting factor. Therefore human data is generally restricted to retrospective studies from both man made and natural disasters. Although there is much controversy surrounding the use of such retrospective data, analysis has revealed some interesting results.

Historical records from Sweden show that individuals born in the early twentieth century had increased mortality due to diabetes if the paternal grandfather was exposed to excess nutrition during his prepubertal slow growth period (Kaati *et al.*, 2002). Similar effects were noted in females when the paternal grandmother had access to abundant nutrition (Pembrey *et al.*, 2006). Similarly studies of the Dutch Hunger Winter have indicated that the offspring of females exposed to famine in the first trimester of pregnancy had reduced birth weight (Stein and Lumey, 2000), an effect which was independent of maternal birth weight. Increased neonatal adiposity and poor health was noted in the second generation following famine exposure (Painter *et al.*, 2008).

1.10.2 Transgenerational programming in Animals

To date, several animal models have backed up data from human studies and demonstrated that environmental factors *in utero* such as diet have far reaching consequences for further generations. Beach *et al*., (1982) was one of the first studies to report a transgenerational effect when feeding Swiss Webster mice a diet that was moderately deficient in zinc from day 7 of gestation. It was noted that the offspring of zinc-deficient mothers had depressed immune function. Furthermore when these offspring were mated the second and third generation were found to have the same depressed immune function as their parents, although not to the same degree as the first generation (Beach, 1982). More recently intergenerational effects have been noted by Drake *et al.*, (2005) who found that reduced birth weight, glucose intolerance and elevated hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity was passed on to the second generation via both the paternal and maternal lines in the dexamethasone-programmed rat (Drake *et al.*, 2005). Torrens and

colleagues (2008) have also recently demonstrated that high blood pressure can be transmitted to the F2 generation following maternal protein restricton during pregnancy. Impaired vasodilatory response to acetylcholine was also noted in these animals suggesting that endothelial dysfunction may contribute to the transgenerational phenotype (Torrens *et al.*, 2008). Transgenerational effects on adiposity in later life (Zambrano *et al.*, 2005), cardiovascular function (Torrens, 2002) and impaired glucose tolerance (Benyshek *et al.*, 2006; Martin *et al.*, 2000; Zambrano *et al.*, 2005) have also been noted in the F1 and F2 generations of rats.

Of prime importance is the report by Anyway and colleagues (2005) that transgenerational effects of male infertility can be transmitted through the male lineage in rats. Maternal exposure to endocrine disruptors such as vinclozolin during the period of sex determination and testis development have been shown to induce defects in offspring fertility due to effects on sperm formation. These effects have been shown to be transferred down the male line to the fourth generation (Anway *et al.*, 2005). Differences in DNA methylation patterns have been noted in these animals (Anway *et al.*, 2005), suggesting epigenetic reprogramming of the germ line has occurred. Increased susceptibility to kidney and prostate disease, tumour development and immune system abnormalities have also been noted (Anway *et al.*, 2006a).

1.10.3 Implications of transgenerational programming

It is well established that maternal undernutrition can establish major effects upon fetal development and risk of adult disease in the F1 generation. Emerging evidence from both human and animal studies is providing evidence that the effects of undernutrition may not be limited to the first generation and as a consequence the

whole field of fetal programming and specifically transgenerational programming is of a major concern to public health. In countries such as India and China where maternal undernutrition has been commonplace for generations, recent economical and nutritional transition towards a more westernised diet, could mean that such countries are on the precipice of unavoidable metabolic disease.

1.11 Aims and hypotheses

The hypotheses to be explored in this thesis are:

- The feeding of a maternal low protein diet during gestation will result in a F1 generation that is hypertensive, and that has a decreased nephron complement and increased adiposity, as has been observed in previous studies.
- The phenotype exhibited by the F1 generation will be passed to a second (F2) and possibly third (F3) generation.
- The postnatal diet will play a critical role in the expression of an obese, hypertensive and renal impaired phenotype and will be a key factor in determining disease risk.
- Prenatal diet will programme adverse phenotypes by altering the expression of key genes associated with tissue development.
- Epigenetic modification of the expression of genes will allow intergenerational transmission of programmed phenotypes.

In order to fully investigate these hypotheses, a single longitudinal study of animals exposed to differing levels of fetal nutrition was performed alongside

behavioural, physiological and chemical analyses which are outlined within chapter two of this thesis.

2.0 Materials and Methods

All chemicals and reagents were of analytical grade or better and were purchased from Sigma-Aldrich (UK), unless stated otherwise in the text. A full list of suppliers is provided in the Appendix (Chapter 8).

2.1 Animal Procedures

The experiments described in this report were performed under license from the Home Office in accordance with the 1986 Animals (Scientific Procedures) Act. The study used rats of the Wistar strain purchased from Charles River (UK). All animals were housed in plastic cages and subjected to a 12 hour light/dark cycle, at a temperature of 20-22˚C. The animals had *ad libitum* access to food and water at all times. Animals were assigned to treatment groups and litters culled by using a random number generator (http://www.graphpad.com/quickcalcs/randomN1.cfm). Treatment groups and cross remained blind to the investigator during all analyses.

2.1.1 Maternal procedure

Figure 2.1 summarises the overall design of this experiment. Twenty virgin female Wistar rats were mated with a single stud male at between 180 and 220g. Upon confirmation of mating by the presence of a semen plug on the cage floor, the rats were allocated to be fed either a 18% (w/w) casein (control) or a 9% (w/w) casein (LP) diet throughout gestation, as described previously (Langley-Evans *et al.*, 1996d). The diets (Table 2.1) were isoenergetic, the difference in protein-derived energy between the two diets being made up with the addition of carbohydrate (starch-sucrose 2:1 w/w). During pregnancy animals were weighed and food intake

was recorded daily. At the time of birth (day 22) animals were transferred to a standard laboratory chow diet (B&K Universal Ltd, UK).

Figure 2.1: Schematic diagram of study design

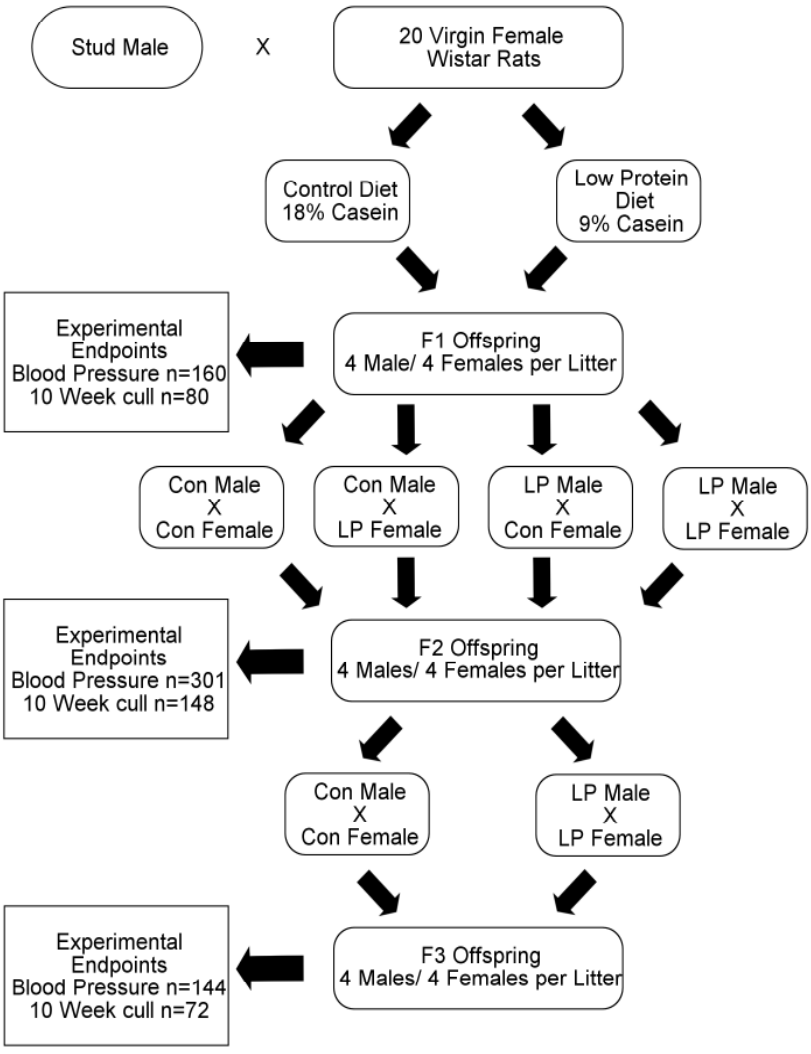


Table 2.1 Formulation of feed given to pregnant rats during pregnancy

Ingredients	CONTROL FEED 18% Casein	LOW PROTEIN FEED 9% Casein
Casein (g/Kg)	180	90
Cornstarch (g/Kg)	425	485
Solkafloc (g/Kg)	50	50
Sucrose (g/Kg)	213	243
Choline Chloride (g/Kg)	2	2
DL-Methionine (g/Kg)	5	5
AIN-76 Minerals (g/Kg)	20	20
AIN-76 Vitamins (g/Kg)	5	5
Corn Oil (g/Kg)	100	100
Energy MJ/Kg	**16.76**	**16.84**

Table 2.1 Diet composition presented as (g/Kg). Diets were prepared by mixing dry ingredients, then binding using water to form a ball of approximately 60g. Diet was then dried at 60°C for 48 hours prior to use (Langley-Evans *et al.*, 1994). The energy content was determined by bomb calorimetry.

2.1.2 F1 offspring Procedure

At birth, individual pups were sexed by measurement of the ano-genital distance and weighed. All litters were randomly culled to 8 pups (4 male and 4 female) to ensure a standard plane of nutrition during suckling. At approximately 3 weeks of age offspring were micro-chipped using the Avid micro-chipping system and allocated into two sub-groups, breeders (B) and nutritional challenge (NC) (2 males and 2 females per group). Breeders and 1 male and 1 female from the NC group were allocated to a standard chow diet (B&K Universal Ltd, UK, Table 2.2). Remaining NC animals were allocated to be fed a high fat diet (Table 2.3), the composition of this diet was identical to that used by Bellinger *et al*, (2004). All animals were housed in single sex groups and had *ad libitum* access to food and water at all times.

At approximately 10 weeks of age, 2 randomly chosen males and 2 randomly chosen females (B sub-group) from each F1 litter were utilised in a breeding programme for production of the F2 generation. The four animals from each litter allocated for breeding, were crossed with animals from another litter in order to produce the four possible crosses from the F1 generation, (Control male x Control female, LP male x LP female, Control male x LP female and LP male x Control female, Figure 2.1). Upon confirmation of mating by the presence of a semen plug on the cage floor the female rats were singly housed and fed standard chow diet until delivery of the litter.

Table 2.2 Formulation of B&K Universal standard chow diet

Ingredients	Composition
Crude Oil (%)	4.73
Crude Protein (%)	18.68
Crude Fibre (%)	3.48
Ash (%)	5.38
NFE (%)	59.73
Digestible Crude Oil (%)	3.99
Digestible Crude Protein (%)	16.53
Gross Energy (MJ/Kg)	16.39
Digestible Energy (MJ/Kg)	14.00
Vitamin A (iu/Kg)	14609.4
Vitamin D_3 (iu/Kg)	1504.1
Vitamin E (mg/Kg)	101.9
Thiamin (mg/Kg)	13.5
Riboflavin (mg/Kg)	11.8
Pyridoxine (mg/Kg)	14.4
Vitamin B_{12} (µg/Kg)	31.5
Vitamin K (mg/Kg)	16.0
Folic Acid (mg/Kg)	2.8
Nicotinic Acid (mg/Kg)	71.3
Pantothenic Acid (mg/Kg)	25.5
Choline (mg/Kg)	1390.2
Inositol (mg/Kg)	1714.0
Biotin (µg/Kg)	357.5
Calcium (%)	0.73
Total Phosphorus (%)	0.70
Magnesium (%)	0.18
Sodium (%)	0.22
Chloride (%)	0.44
Potassium (%)	0.69
Iron (mg/Kg)	87.0
Copper (mg/Kg)	18.3
Manganese (mg/Kg)	86.9
Zinc (mg/Kg)	92.5
Cobalt (µg/Kg)	456.8
Iodine (µg/Kg)	1840.8
Selenium (µg/Kg)	391.8
Lysine (%)	1.10
Methionine (%)	0.33
Linoleic Acid (%)	2.04
Linolenic Acid (%)	0.18

Table 2.2 B&K diet composition, Data obtained directly from B&K Universal Limited.

Table 2.3 Formulation of diet given to high fat-fed animals

Ingredients	HIGH FAT FEED
Casein (g/Kg)	200
Cornstarch (g/Kg)	220
Lard (g/Kg)	295
Sucrose (g/Kg)	100
Solkafloc (g/Kg)	50
DL-Methionine (g/Kg)	10
AIN-76 Minerals (g/Kg)	10
AIN-76 Vitamins (g/Kg)	5
Corn Oil (g/Kg)	100
Energy MJ/Kg	**25.12**

Table 2.3 Composition of diet used in high-fat feeding (Bellinger *et al.*, 2004). The high fat diet ingredients were mixed dry and stored at -40°C until needed. The energy content was determined by bomb calorimetry.

2.1.3 F2 offspring Procedure

At birth, pups were sexed and weighed. All litters were randomised and then culled to 8 pups (4 male and 4 female) to ensure a standard plane of nutrition during suckling. At approximately 3 weeks of age offspring were micro-chipped and randomly allocated into B and NC sub-groups as described above. Breeders and 1 male and 1 female from the NC group were allocated to a standard chow diet (B&K Universal Ltd, UK). Remaining NC animals were allocated to be fed a high fat diet. The animals were housed in single sex groups and had *ad libitum* access to food and water at all times.

At approximately 10 weeks of age, 1 male and 1 female (B sub-group) from each litter were crossed with animals from another litter in order to produce the following crosses based upon the dietary exposures of their parents in the F1 generation: Control male x Control female and LP male x LP female (Figure 2.1). Upon confirmation of mating by the presence of a semen plug on the cage floor the

female rats were singly housed and fed standard chow until delivery of the litter. Male rats were placed back into single-sexed housing with their littermates, until studied later in life.

2.1.4 F3 offspring Procedure

At birth, pups were sexed and weighed. All litters were randomised and then culled to 8 pups (4 male and 4 female) to ensure a standard plane of nutrition during suckling. At approximately 3 weeks of age offspring were micro-chipped and allocated to a standard chow diet (B&K Universal Ltd, UK). One male and one female animal were randomly allocated to be fed a high fat diet. The animals were housed in single sex groups and had *ad libitum* access to food and water at all times.

2.1.5 High Fat Feeding Procedure

Rats (where possible 1 male and 1 female offspring from each NC sub-group per litter, in each generation) were randomised to a high-fat feeding protocol to assess the impact of this nutritional challenge upon subsequent weight gain and body composition, from approximately 4 weeks of age. The high-fat diet was identical to that used in our previous work (Erhuma *et al.*, 2007b) and contained 29.5% fat (w/w) in the form of lard, and comprised 20% (w/w) protein (casein). The gross energy content of the diet was 25.12 MJ/Kg (Table 2.3). A further group (consisting of 1 male and 1 female offspring from each NC group per litter, in each generation) were fed a standard laboratory chow diet as a control (Table 2.2). This diet contained 19% protein, 4.7% fat and had a gross energy content of 16.39 MJ/Kg. All animals from

this group were housed in pairs and provided with *ad libitum* access to high fat diet or standard laboratory chow. At 5, 7 and 9 weeks of age animals were singly housed and food intake and body weight was monitored for a period of 3 days. Food intake data is presented corrected for body weight, as in our previous studies of appetite in this model (Bellinger *et al.*, 2004). This corrects for any influence of body weight upon food intake, and allows comparison between sexes.

2.2 Aging protocol

Male animals used in production of F2 and F3 generations were retained until 6 months old. Male and female F2 rats not used in the production of the F3 generation were retained until 1-year-old.

2.3 Culling of animals and sample collection

At approximately 10 weeks of age the NC group of animals was killed to coincide with matings for the next generation. Animals were culled using a rising concentration of CO2 and cervical dislocation. Liver, heart, kidneys, lungs, spleen, thymus, hippocampus, hypothalamus, gonadal fat and perirenal fat were rapidly removed and accurately weighed, then snap-frozen in liquid nitrogen and stored at -80°C for up to 18 months until used for further analysis. The left kidney was fixed in formalin for later determination of nephron number.

2.4 Chemical Analyses

2.4.1 Coefficients of variation (COV)

For all chemical analyses the intra-assay coefficient of variation was calculated to assess assay variation within each run/batch. The inter-assay coefficient of variation was calculated to assess day-to-day variation between runs/batches.

$$\text{COV} = \frac{\text{Standard Error of Mean}}{\text{Mean}} \times 100\%$$

2.4.2 Preparation of plasma

Blood samples were collected and centrifuged at 13000rpm, 4°C for 10 minutes in order to separate plasma. The plasma was subsequently removed and stored at -40°C for up to 6 months until used for analyses.

2.4.3 Determination of circulating metabolites

2.4.3.1 Cholesterol and Triglyceride Assays

Total circulating plasma cholesterol and triglycerides were assayed using commercially available kits (ThermoTrace, Noble Park, Victoria, Australia), according to the manufacturer's instructions. A standard curve was produced by making serial dilutions of the cholesterol/ triglyceride standards contained within the kit. 10µl of sample (plasma) or diluted standard was transferred in duplicate to a 96-well plate, 200µl of cholesterol/triglyceride assay reagent was then pipetted into each

well. The plate was then allowed to incubate at 37°C for 15 minutes. The 96-well plate was then read at an absorbance of 550nm (reference wavelength 630nm) using BIO-RAD 680XR Microplate Reader and BIO-RAD Microplate manager software version 5.2.1, (BIO-RAD, UK). Assay linearity; Cholesterol (20 mmol/L), triglycerides (10 mmol/L). Assay sensitivity; Cholesterol (62 ΔmA per mmol/L), triglycerides (0.158 ΔA per mmol/L). The inter- and intra-assay coefficients of variation were 4.4% and 3.1% respectively for the triglyceride assay and 2.2% and 3.2% respectively for the cholesterol assay.

2.4.3.2 Glucose Assay

Plasma glucose concentrations were determined in samples from non-fasted animals, using an adaptation of the glucose oxidase method (Trinder, 1969). A standard curve was produced by making serial dilutions of the glucose standard (0-2mg/ml glucose). Samples were diluted 1:5 with phosphate buffer and loaded with standards in duplicate onto a microtitre plate in 10µl quantities, 200µl of glucose reagent (Section 8.2.1) was then added to each well. The plate was then incubated at 37°C for 15 minutes and then read at an absorbance of 620nm using Magellan, Version 4.0 software and plate reader (Tecan, Sunrise). The inter- and intra-assay coefficients of variation were 7.2% and 3.3% respectively.

2.4.3.3 Insulin (non-fasted)

Plasma insulin concentrations were determined in a 96-well plate using the Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem Inc, USA). All reagents and samples were allowed to reach room temperature prior to analysis. Each well was

washed twice with 300µl of washing buffer, aspirated manually and blotted dry. 50µl of Guinea Pig Anti-Insulin and 45µl of diluent was added to each well before 5µl of sample or standard was added to each well in duplicate. The microplate was then covered and allowed to incubate at 4°C overnight (16-20 hours). The plate was aspirated and washed three times with 300 µl of washing buffer per wash and blotted dry to remove any unbound materials. 100 µl of Anti-Guinea Pig Antibody Enzyme Conjugate was added to each well. The microplate was then covered and allowed to incubate at room temperature for 3 hours. Each well was then washed five times with 300 µl of washing buffer and blotted dry after each fresh addition of buffer to remove any unbound materials. 100 µl of Enzyme substrate solution was added to each well and allowed to react for 30 minutes at room temperature (avoiding exposure to light). The reaction was then stopped with the addition of 50 µl of Enzyme Reaction Stop Solution per well. The plate was then immediately read at a wavelength of 492nm using the Magellan Version 4.0 computer package and plate reader (Tecan, Sunrise). Mean concentrations were calculated from the duplicated raw data quantified from the rat insulin standard curve (range 0-10ng/ml) which used seven points derived from serial dilutions. The inter- and intra-assay coefficients of variation for this assay were 9.5% and 8.2% respectively.

2.5 Physical and behavioural studies

2.5.1 Activity Monitoring

Locomotor activity was determined using a Linton AMIU53 Infrared Activity Monitor (Linton Instruments, Diss, UK). The monitor consists of a grid of 48 infrared beams and sensors set on two levels. Beams may be broken by animal movement in the X-Y plane or by rearing in the Z plane. Events were recorded by the

AMlogger software for a total period of 90 minutes. The first 30 minutes were used to assess the response to a novel environment since animals were transferred from their home cage to the test cage. After 30 minutes they were regarded as habituated to the test conditions. Locomotor activity was only assessed during the 12-hour light phase. Within the test, three types of movement were recorded:

Activity = Breaking any beam on the lower level within 1 second.

Mobility = Breaking More than one beam (50mm apart) on the lower level, within 1 second.

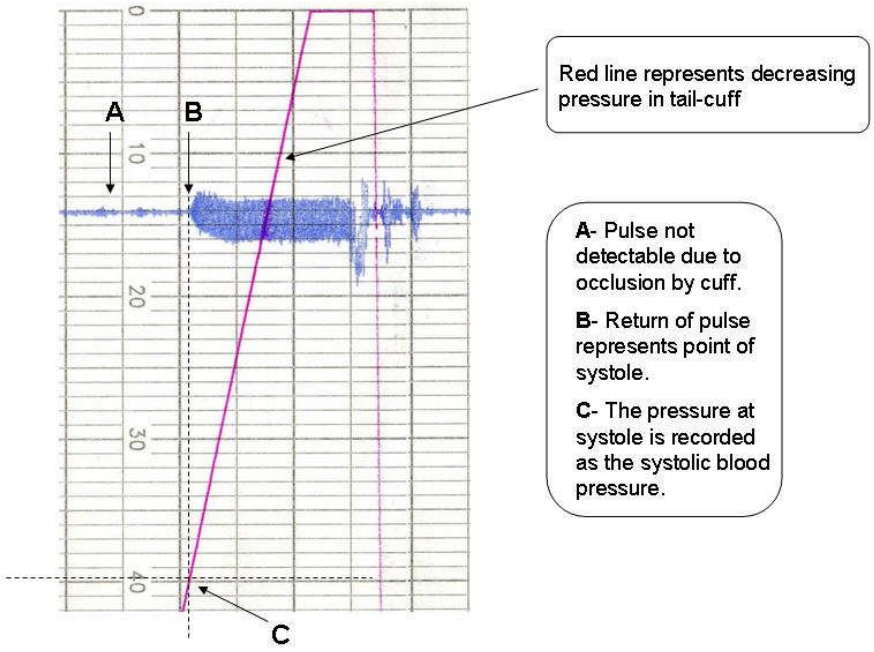
Rearing = Breaking a beam on the upper level recorded in 1 second intervals.

2.5.2 Determination of Blood Pressure

Blood pressure was determined in all animals using an indirect tail cuff method, as described previously (Sherman and Langley-Evans, 1998). Measurements were made using an IITC Life Sciences Model 229 Blood Pressure amplifier/ pump (IITC Inc, Woodlands CA, USA). This instrument allows measurements at a temperature of 27°C, thereby avoiding heat-stress of the animals. The unheated animal tail-cuff method has been previously shown to be an accurate and sensitive technique for the non-invasive measurement of systolic blood pressure in the conscious rat (Ibrahim *et al.*, 2006; Kubota *et al.*, 2006). All rats were housed at 27°C, in the room where the measurements were made for at least 2 hours prior to testing. Measurements were made at the same time of day following standard

procedures to minimise variation due to diurnal changes in blood pressure. Restraint tubes and tail-cuff sizes were selected to be appropriate for the weight and size of the animal and the tail-cuff was placed in the standard position at the base of the tail. In order to minimise the impact of stress during the procedure (Tonkiss *et al.*, 1998), animals were restrained for no more than 5 minutes at any one time, after the procedure, the animals were placed directly back in to the home cage with their litter mates. No training of the rats to the restraint conditions was needed (Sherman and Langley-Evans, 1998). Blood pressure was determined at 4, 6 and 8 weeks of age. Measurements were taken in triplicate and an average value was derived for each animal (Figure 2.2). The inter-assay coefficients of variation (Values obtained from the same set of animals on different days) and intra-assay coefficients of variations were 1.39% and 1.42% respectively.

Figure 2.2 Example blood pressure Trace.



2.5.3 Nephron Number Determination

Nephron number was determined using an adaptation of the acid maceration method of Welham *et al*.,(Welham *et al.,* 2002). Although determination of nephron complement via stereology (Bertram, 2001) is the gold standard method of analysis, we used maceration in the current study due to the high throughput required (253 samples). Our results and overall effect matched those obtained via stereology in previous studies of the LP diet in the F1 generation (Zimanyi, 2000). Formalin fixed kidneys were weighed, cut in half and one portion was then incubated in 1 mol/L hydrochloric acid at 37°C for 30 minutes. Acid was removed and replaced with 5 ml of 50mM phosphate buffered saline (PBS; pH 7.4). The tissue was then homogenised using a bench top homogeniser (Polytron) and a further 5ml of PBS was added to give a final volume of 10ml. The sample was mixed thoroughly by inversion and a 20µl sample was taken and placed on a microscope slide and overlain with a cover slip. A ×10 objective lens was used to count all glomeruli in the sample. This was carried out in triplicate for each tissue sample. The values obtained were averaged and used to calculate the total number of glomeruli per kidney using the following equation.

$$\text{Total Nephron number} = \frac{\text{Kidney weight} \times \text{Average count}}{\text{Sample weight}} \times 500$$

The inter- and intra-assay coefficients of variations were 2.0% and 2.2% respectively.

2.6 Analyses of gene expression

2.6.1 RNA extraction and sample preparation

Rat kidneys were removed at the time of cull, snap-frozen in liquid nitrogen and stored at -80°C until required for further analysis. Total RNA was isolated from frozen kidney samples using the Trizol method (Invitrogen, UK). The RNA was treated with DNase (Promega, UK) and subjected to phenol-chloroform extraction and ethanol precipitation. RNA concentration was determined spectrophotometrically (Nanodrop) and RNA integrity was confirmed on a 1% agarose gel via electrophoresis.

2.6.1.1 Trizol™ extraction

Frozen rat kidneys stored at -80°C were transferred to a pestle and mortar. Liquid nitrogen was poured over the kidney sample and it was ground into a fine powder. Approximately 100mg of frozen crushed tissue was weighed into autoclaved 15ml glass tubes. 1ml of Trizol™ reagent (Invitrogen, UK) was added, and tissue was then homogenised using a Polytron™ homogeniser, which was rinsed between samples using ddH_20. The homogenate was pipetted into autoclaved eppendorf tubes and centrifuged at 12000 x g for 10 minutes at 4°C. Supernatant was then transferred to a fresh eppendorf tube, to remove cell debris and incubated at room temperature (18-30°C) for 5 minutes. 200µl chloroform (Fisher, UK) was added to each sample and shaken vigorously for 15 seconds to ensure mixing of the contents. Each sample was then incubated at room temperature for 3 minutes and centrifuged at 12000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh eppendorf tube

and 500 µl of isopropyl alcohol was added to precipitate the RNA. The samples were vortexed and incubated at room temperature for 10 minutes. The samples were then centrifuged at 12000 x g for 10 minutes at 4°C. The supernatant was then removed and 1ml of 75% ethanol was added to the resulting RNA pellet and vortexed.

2.6.1.2 DNase Treatment

RNA was treated with DNase to ensure any remaining DNA contamination, which could affect results during later analysis, was removed. Each sample (RNA and ethanol) was centrifuged at 13000 x g for 15 minutes at 4°C to produce a pellet. The ethanol was then removed and the pellet allowed to air-dry for 5 minutes, before being redissolved in 40 µl RNase free water (Promega, UK), 5 µl DNase 10x buffer (Promega, UK) and 5 µl DNase (Promega, UK). The samples were then vortexed to ensure thorough mixing of components and spun down to collect the mixture at the bottom of the eppendorf tube, and then incubated at 37°C for 30 minutes. 150 µl of RNase free water and 200 µl of phenol/chloroform/isoamyl alcohol (25:24:1), (Fisher, UK) were added to each sample, the mixture was again vortexed and centrifuged at 13000 x g for 5 minutes at 4°C to allow phase separation. The resulting aqueous layer containing RNA was pipetted into a fresh eppendorf and 15 µl of 3M sodium acetate pH 5.5 and 375 µl of 100% ethanol was added to each tube. The resulting mixtures were allowed to precipitate overnight at -80°C. The following morning samples were centrifuged at 13000 x g for 15 minutes at 4°C to pellet the RNA The supernatant was removed and the RNA pellet was washed by the addition of 1ml 75% ethanol. The samples were centrifuged for a further 5 minutes. The ethanol was then removed and the RNA pellet was allowed to air-dry for 5 minutes,

before resuspending in 70 µl of RNase free water by passing through a pipette several times.

2.6.1.3 RNA Quantification

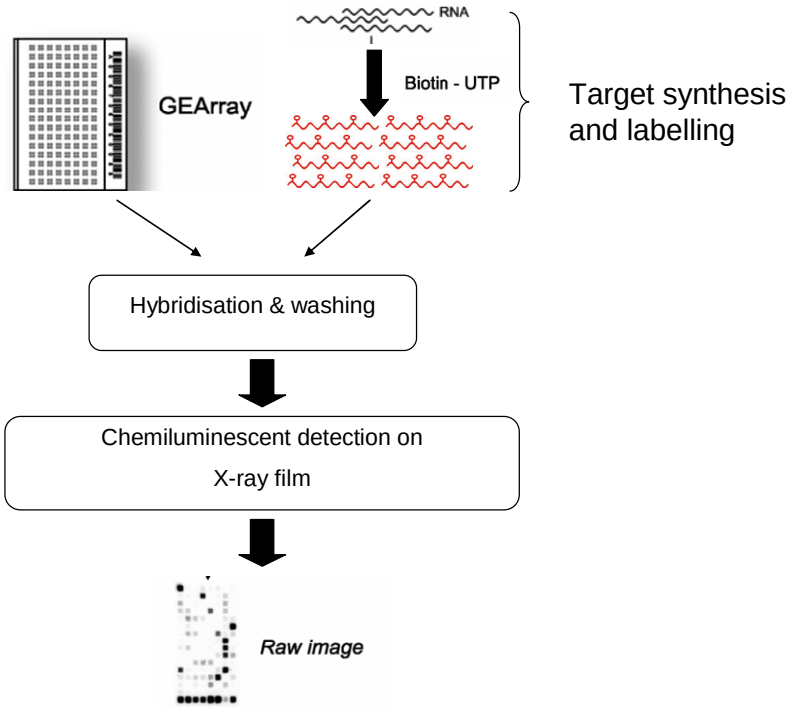
RNA concentration was quantified using a Nanodrop® spectrophotometer (Thermo, USA). 1 µl of each sample was placed onto the Nanodrop® pedestal and quantified in duplicate using the Nanodrop® software provided with the spectrophotometer. The pedestal was then wiped clean with a sterile laboratory tissue, RNase free water was used as a blank. The Nanodrop® allows accurate and reproducible quantification of nucleic acid samples upto 3700 ng/µl without dilution, in samples as small as 1µl. The instrument determines concentration values as well as 260/280 and 260/230 ratios. RNA samples were then diluted to 500ng/µl by the addition of RNase-free water.

2.6.2 Pathway focussed DNA microarray:

Gene expression was analysed via DNA MicroArray analysis using the pathway focussed Oligo GEArray® for rat endiothelial cell biology ORN-015.2 (SuperArray, USA). Microarray technology utilises RNA to determine the expression levels of several hundred or thousand genes simultaneously. Microarrays are composed of a number of gene-specific, nucleic acid probes that are immobilised on a solid membrane. During the microarray procedure (Figure 2.3), RNA is converted to labelled cDNA/cRNA and then hybridised to the immobilised nucleic acid probes on the membrane, the labelled cDNA/cRNA bound to each gene-specific spot is

detected using a chemiluminescent step. The resulting signal produced at each spot on the membrane is representative of the amount of expression in the original RNA sample.

Figure 2.3 Overview of Microarray process.



2.6.2.1 cDNA Synthesis

2.6.2.1.1 Preparation of Annealing Mixture

cDNA synthesis was completed using the recommended TrueLabeling-AMP™2.0 kit (SuperArray, USA). True-labelling primer, 5X cDNA synthesis buffer and RNase-free water were allowed to thaw and were subsequently mixed and collected at the bottom of the tube via a brief spin in a microcentrifuge. The reagents were then placed on ice prior to beginning the procedure. 1 µg of RNA and 1 µl of True-labelling primer were combined in a sterile PCR tube with the addition of

RNase-free water to a final volume of 10 µl. Contents of the tubes were mixed by passing through a pipette tip several times and then briefly centrifuged to collect the mixture at the bottom of the tube. The tubes were then incubated at 70°C for 10 minutes followed by a brief centrifugation to collect mixture at the bottom of the tube. The tubes were immediately placed on ice.

2.6.2.1.2 Preparation of cDNA Synthesis Master Mix

4 µl of RNase-free water, 4 µl of 5X cDNA synthesis buffer, 1 µl RNase Inhibitor and 1 µl of cDNA Synthesis Enzyme Mix were combined in a sterile eppendorf tube to achieve a final volume of 10 µl per reaction. The master mix was mixed gently with a pipettor, followed by a brief centrifugation to collect the mixture at the bottom of the tube. The cDNA Synthesis Master Mix was then placed on ice.

2.6.2.1.3 cDNA Synthesis Reaction

10 µl of cDNA Synthesis Master Mix was added to each tube containing 10 µl of Annealing Mixture and mixed gently with a pipettor followed by a brief centrifugation to collect mixture at the bottom of the tube. The mixture was then incubated at 42°C for 50 minutes followed by 75°C for 5 minutes, the mixture was then cooled and held at 37°C. The mixture was then briefly centrifuged and returned to 37°C until the addition of the Amplification Master Mix.

2.6.2.2 cRNA Synthesis, Labeling, and Amplification

2.6.2.2.1 Preparation of Amplification Master Mix

2.5X RNA Polymerase buffer was warmed to room temperature and vortexed to resuspend any precipitate. The buffer was then briefly centrifuged to collect material at the bottom of the tube. RNA polymerase enzyme was removed from -20°C storage and transferred to ice just before use.

16 µl of 2.5X RNA Polymerase buffer, 2 µl of biotinylated-UTP, 10mM (Roche, UK) and 2 µl of RNA Polymerase were combined at room temperature in a sterile eppendorf to a final volume of 20 µl per reaction. The master mix was mixed gently with a pipettor to ensure a homogenous solution and then briefly centrifuged to collect mixture at the bottom of the tube.

2.6.2.2.2 cRNA Synthesis Reaction

20 µl of Amplification Master Mix was added to each tube containing 20 µl of cDNA Synthesis Reaction, then gently mixed with a pipettor, briefly centrifuged and incubated in a PCR block at 37°C overnight.

2.6.2.3 cRNA Purification

2.6.2.3.1 Binding the cRNA to the Spin Column

60 µl of RNase-free water was added to each cRNA synthesis reaction tube to give a final volume of 100 µl per reaction. Each reaction was then transferred to a 1.5ml RNase-free eppendorf and 350 µl of lysis & binding buffer was added to each reaction mixture and mixed gently with a pipettor. 350 µl of ACS-Grade 100%

ethanol (Fisher, UK) was then added to each reaction mixture, which was then mixed with a pipettor and immediately transferred to the centre of a spin column. The spin column was then centrifuged for 30 seconds at 8000 x g. The flow-through in the collection tube was then discarded, and the spin column was placed in a fresh collection tube.

2.6.2.3.2 Washing the Spin Column

600 µl of washing buffer (containing ethanol) was added to each spin column, the spin column was then centrifuged at 8000 x g for 30 seconds. The flow-through was discarded and the spin column was then put into a fresh collection tube. 200 µl of washing buffer was then added. The columns were then centrifuged at 11000 x g for 1 minute, the flow-through discarded and the spin column put back into the collection tube and centrifuged at 180° from its original orientation for 2 minutes at 11000 x g.

2.6.2.3.3 Eluting the cRNA from the Spin Column

Each spin column was transferred to its own elution tube and 50 µl of room temperature RNase-free 10mM Tris-HCl Buffer (pH 8.0) was added to the centre of each spin column. The spin columns were then incubated at room temperature for 2 minutes and subsequently centrifuged at 8000 x g for 1 minute. The purified cRNA was then stored on ice until quantification.

2.6.2.3.4 cRNA Quantification and Quality Assesment

Quantification of cRNA was performed using a Nanodrop® spectrophotometer, as previously described (Section 2.6.1.3). The yield was determined using the following equation.

Yield = Concentration (µg/µl) × 50 µl

2.6.2.4 Array: Pre-hybridisation

5ml of deionised water was added to each hybridisation tube. The tubes were then allowed to sit inverted for 5 minutes in order to pre-wet the array membrane. The GEAhyb Hybridisation Solution was warmed to 60°C and the bottle inverted several times to allow complete dissolution of the buffer components. The deionised water was then discarded from the hybridisation tube and 2ml of pre-warmed GEAhyb Hybridisation Solution was added to each tube. The tube was then vortexed briefly. The hybridisation tubes were then placed in the hybridisation oven and pre-hybridised at 60°C for 2 hours with continuous, but slow, agitation (5-10rpm).

2.6.2.5 Array: Hybridisation

To prepare the target hybridisation mixture, 2µg of biotin-labeled cRNA target prepared using the True-Labeling-AMP 2.0 kit (section 2.6.2.1-2.6.2.3) was added to an eppendorf containing 750µl of pre-warmed GEAhyb Hybridisation Solution, mixed well via a pipettor and kept at 60°C. The pre-hybridisation solution was discarded from the hybridisation tube and the Target Hybridisation Mix containing

the labelled cRNA target was added to the tube. The hybridisation tube was then allowed to hybridise overnight at 60°C in the hybridisation oven with continuous, slow agitation (5-10rpm).

2.6.2.6 Array: Washing

The target hybridisation mix from the hybridisation tube was poured into a sterile eppendorf. 5ml of Wash Solution 1 was added to each hybridisation tube. The hybridisation tube was then vortexed and placed back into the hybridisation oven and the array membrane was washed at 60°C for 15 minutes with fast agitation (28rpm). The wash solution was then discarded and 5ml of Wash Solution 2 was added to each hybridisation tube. The tube was then vortexed and placed back in the hybridisation oven, the membrane was washed for exactly 15 minutes at 60°C, with fast agitation (28rpm). Wash solution 2 was then discarded and the cap was placed back on the hybidisation tube to prevent the membrane from drying out. The tube was allowed to cool to room temperature.

2.6.2.7 Array: Chemiluminescent Detection

2ml of GEAblocking Solution Q was added to each hybridisation tube, the tube was then vortex briefly and allowed to incubate at room temperature in the hybridisation oven with fast agitation, for 40 minutes. The GEAblocking Solution Q was then discarded and 2ml of dilute AP-SA Buffer was added. The tube was then vortexed and incubated in the hybridisation oven at room temperature for exactly 10 minutes with gentle agitation. The membrane was then washed four times with 4ml of 1X Buffer F, for 5 minutes, with gentle agitation. The last wash was then

discarded and the membrane was then washed twice with 3ml of Buffer G. Once complete, the second wash was discarded and 1ml of CDP-Star Chemiluminescent substrate was added to each hybridisation tube. The tubes were then placed into the hybridisation oven and allowed to incubate at room temperature for 5 minutes with gentle agitation. The membrane was then removed from the hybridisation tube using sterile forceps. The excess CDP-Star solution was removed from the membrane by blotting on clean absorbent paper and the membrane was then placed into a re-sealable plastic bag for image acquisition.

2.6.2.8 Array: Image Acquisition

Image acquisition was carried out by exposure to X-ray film (Amersham Hyperfilm ECL, UK) within 5 minutes of CDP-Star treatment. Exposure times of 1, 2, and 5 minutes were imaged. Once dry the X-ray film images were photographed using a CCD camera (BIO-RAD Fluor-S™ MultiImager and BIO-RAD Quantity One software version 4.2.1, UK) and saved as a 16 bit TIFF image, the images were then uploaded to the specially designed web-based GEArray Expression Analysis Suite (http://geasuite.superarray.com, SuperArray, USA) for analysis. Any gene with either a two-fold increase in expression or a 50% decrease in expression compared to control samples was considered for further analysis.

2.7 DNA Methylation Assay

2.7.1 DNA Isolation (Qiagen, DNeasy: Animal Blood and Tissue kits)

25mg of crushed and frozen rat kidney was placed in a 1.5ml eppendorf, 180 µl of ATL Buffer and 30 µl of Proteinase K were then added, the mixture was then

incubated at 56°C and vortexed every 30 minutes until completely lysed (4 hours). After lysis each sample was vortexed for 15 seconds, then 200 µl of Buffer AL and 200 µl of 100% ethanol (Fisher, UK) was added to each sample. The sample was then vortexed thoroughly. Each sample was then pipetted into a DNeasy Mini spin column (Qiagen) in a 2ml collection tube and centrifuged at 6000 x g for 1 minute. The flow-through was then discarded and the spin column was placed in a fresh collection tube. 500 µl of Buffer AW1 was then added to the spin column, which was then centrifuged at 6000 x g for 1 minute. The flow-through was discarded and the spin column was placed in a fresh collection tube. 500 µl of Buffer AW2 was then added to each spin column which was then centrifuged at 20000 x g for 3 minutes. The flow-through was discarded and the spin column was carefully transferred to a sterile 1.5ml elution tube. 200 µl of elution buffer AE was then added to each spin column. The column was allowed to incubate at room temperature for 1 minute, before being centrifuged at 6000 x g for 1 minute. DNA quality and quantity was analysed using the Nanodrop® spectrophotometer as described in section 2.6.1.3. DNA samples were then diluted to 50ng/µl.

2.7.2 Cytosine extension assay

The cytosine extension assay (Maloney *et al.*, 2007; Pogribny *et al.*, 1999) is a method for analysing global DNA methylation The assay utilises a methylation sensitive restriction endonuclease (*Hpa*II) and its isochizomer *Msp*I, combined with a single nucleotide extension with radiolabeled [3H]dCTP. Both enzymes recognise the restriction site 5‘-CCGG-3' , however *Hpa*II is methylation sensitive and therefore only cuts unmethylated DNA whereas *Msp*I cuts DNA regardless of its methylation status. The 5‘-CCGG-3' restriction site recognised by both *Hpa*II and

*Msp*I occur randomly throughout the genome. Once radio-labelled the difference between the two restriction endonucleases can be used to work out global methylation.

1µg of genomic DNA was digested with the restriction enzymes *Msp*I and *Hpa*II, DNA was also mock digested with water as an internal control. Each digestion was done in triplicate in a mixture of 0.4µl bovine serum albumin (BSA),(Promega, UK), 6µl of the appropriate restriction enzyme buffer (Promega, UK), 3 µl of appropriate restriction enzyme (Promega, UK) or water and 0.6 µl of ddH20 (Sigma, UK) to make a final volume of 30 µl per reaction. The reaction was incubated overnight at 37°C. 15 µl of each digested DNA sample was then transferred to a fresh eppendorf where 40µl of cytosine extension master mix containing 10µl DNA polymerase buffer (Promega, UK), 4µl of 25mM magnesium chloride (Promega, UK), 0.25µl DNA Polymerase (Promega, UK), 25.6744µl ddH_20 and 0.0756µl of 250µCi [^{3}H]dCTP, specific activity 59.0 Ci/mmol, (Amersham, UK) per reaction. The mixture was then incubated at 56°C for 1 hour before being cooled on ice for 5 minutes. 10µl of each reaction was then transferred to DE-81 ion exchange filters (Whatman, UK) and washed three times with 300µl of 0.5M sodium phosphate buffer, pH 7.0. The filters were then washed with 150µl of 75% ethanol (Fisher, UK) and allowed to air dry for 30 minutes. The filters were then transferred to scintillation vials where 4ml of scintillant (Perkin-Elmer, UK) was added. The incorporation of [^{3}H]dCTP was then counted using a TRI-CARB 2100TR liquid scintillation analyzer (Perkin-Elmer, UK). The counts for the mock digestion were considered as the background count for that DNA sample and were subtracted from both the *Msp*I and *Hpa*II counts. In order to normalise the data for inconsistent amounts of starting material, for each sample, the ratio between the *Hpa*II and *Msp*I

counts were determined as an index of DNA methylation. The inter- and intra-assay coefficients of variation for *Msp*I and *Hpa*II were 0.85%, 2.34%, 0.60% and 1.81% respectively.

2.8 Quantitative Reverse Transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was used to detect the expression of mRNA for Matrix metallopeptidase 9 (MMP9), Granzyme B (GZMB), Interleukin 7 (IL-7), Endothelial cell growth factor 1 (ECGF1) and Endothelin receptor type A (EDNRA) mRNA in rat kidneys. This was done as a two step process therefore allowing both the reverse transcriptase step and the polymerase chain reaction step to be optimised independently. Rat Kidneys were removed at time of cull as described previously (section 2.6.1) and stored at -80°C until further analysis was required. Total RNA was isolated from frozen tissues via the Trizol method (section 2.6.1.1), RNA was DNase treated (section 2.6.1.2) and quantified using the Nanodrop spectrophotometer (section 2.6.1.3), RNA integrity was confirmed on a 1% agarose gel via electrophoresis.

2.8.1 Annealing oligo primer to RNA (Reverse Transcriptase Reaction)

The reverse transcriptase reaction synthesises complementary strand DNA (cDNA) from single stranded RNA. 5.33µl of 0.1µg/µl DNase treated total RNA, 1.06µl of Random Primers (Promega, UK) and 9.6µl of ddH_20 (Sigma, UK) was added to a Thermo-Fast® 96 well detection plate (ABgene) to make a total volume of 16 µl per well, the detection plate was then sealed and placed in a PCR block

(Applied Biosystems, UK) and incubated at 70°C for 5 minutes followed by 4°C for 15 minutes. The detection plate was then put on ice and 1µl of each reaction on the detection plate was transferred to a second detection plate which was then sealed and stored on ice for –RT analysis (section 2.8.2.1), the remaining 15µl was used for +RT analysis (section 2.8.3.1).

2.8.2 -RT Reaction (Contamination Control)

The –RT reaction was used to confirm there was no contamination of genomic DNA within the RNA. 14µl of ddH_20 (Sigma, UK) was added to each well containing 1 µl of reaction mixture from the annealing of the oligo primer to RNA (section 2.8.1). For multiple reactions a master mix (Table 2.4) was made and then mixed and 10µl added to the diluted primer/RNA mix in each well of the detection plate.

Table 2.4 –RT Mastermix (All reagents supplied from Promega, UK)

Reagent	**Volume**	**Final concentration**
MMLV Reverse Transcriptase buffer (×5)	5 µl	-
Nucleotides (dNTPs) 10mM of each	1.25 µl	0.5mM
RNase inhibitor	0.5 µl	25U
MMLV Reverse Transcriptase	No Enzyme	
ddH_20 (RNase/DNase free)	3.25 µl	-
Total volume	10 µl	-

The contents of each well (final volume 25 µl) were mixed gently with a pipette, sealed and incubated at room temperature for 10 minutes before being placed in a PCR block (Applied Biosystems, UK) and incubated at 42°C for 60 minutes.

After incubation each well within the detection plate was diluted by adding 75 µl of ddH_20 (Sigma, UK), mixed with a pipette, labelled as "Stock -RT" and stored at -20°C.

2.8.2.1 -RT Analysis

5µl of each –RT reaction was transferred to a separate well in a 384 well plate detection plate. A probe mastermix (Table 2.6) containing β-actin primers and probes was made, vortexed and spun down. 10µl of the probe mastermix was then added to each well containing 5µl of –RT reaction. The plate was then sealed and spun down, and was then analysed using a standard probe run protocol on the Lightcycler 480 and software version 1.5 (Roche, UK). Any samples showing positive fluorescence were noted and omitted when pooling cDNA (section 2.8.4).

2.8.3 +RT Reaction

The annealed primers in the remaining 15 µl of reaction mixture (Section 2.8.1) are extended using the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. For multiple reactions a master mix (Table 2.5) was made and 10µl added to the primer/RNA mix in each well of the detection plate.

Table 2.5 +RT Mastermix. (All reagents supplied from Promega, UK)

Reagent	**Volume**	**Final concentration**
MMLV Reverse Transcriptase buffer (×5)	5 µl	-
Nucleotides (dNTPs) 10mM of each	1.25 µl	0.5mM
RNase inhibitor	0.5 µl	25U
MMLV Reverse Transcriptase	1 µl	200U
ddH_20 (RNase/DNase free)	2.25 µl	-
Total volume	10 µl	-

The contents of each well (final volume 25 µl) were mixed gently with a pipette, sealed and incubated at room temperature for 10 minutes before being placed in a PCR block (Applied Biosystems, UK) and incubated at 42°C for 60 minutes. After incubation each well within the detection plate was diluted by adding 75 µl of ddH_20 (Sigma, UK), mixed with a pipette, labelled as "Stock +RT" and stored at -20°C.

2.8.3.1 +RT Analysis

5µl of each cDNA sample synthesised during +RT step (section 2.8.3) was transferred to a separate well in a 384 well detection plate. A probe mastermix (Table 2.6) containing β-actin primers and probes was made, vortexed and spun down. 10µl of the probe mastermix was then added to each well containing 5µl of +RT reaction. The plate was then sealed and spun down and then analysed using a standard probe run protocol on the Lightcycler 480 and software version 1.5 (Roche, UK). Any samples which had a fluorescence value either two standard deviations higher or lower than the mean fluorescence were noted and omitted when pooling cDNA (section 2.8.4).

2.8.4 Production of pooled cDNA

25µl of each sample that was not omitted during the –RT (section 2.8.2.1) and +RT (Section 2.8.3.1) analysis was pooled together to from a Pooled cDNA sample that was then used to generate a standard curve for each gene of interest during qPCR analysis.

2.8.5 Sample dilution

The pooled cDNA produced in section 2.8.4, was diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128, 5µl of each dilution was transferred in triplicate to a 384 well detection plate, 10µl of β-actin probe mastermix was then added to each well as previously described (section 2.8.2.1). The plate was then sealed and spun down. The plate was then analysed using a standard probe run protocol on the Lightcycler 480 and software version 1.5 (Roche, UK). The dilution factor of 1:8 was found to be the optimum dilution for sample analysis. All + RT samples were then diluted 1:8 with molecular grade ddH_20 (Sigma, UK).

2.8.6 Quantitative Polymerase Chain Reaction (qPCR)

This reaction used the diluted cDNA from section 2.8.5. The Roche lightcycler® 480 was used to amplify cDNA samples. The Lightcycler system is a fluorophore-based real time PCR system which allows highly accurate qualitative and quantitative detection of varying mRNA levels in samples via fluorescence. The fluorescence is produced via a probe which is a single-stranded oligonucleotide complementary to a segment of nucleotides within the cDNA template located

between two primers. The probe has a fluorophore (eg 6-carboxyfluorescein (FAM)) on the 5' end of the probe and a quencher (e.g. tetramethylrhodamine, (TAMRA)) on the 3' end. The close proximity of the quencher and fluorophore inhibits fluorescence. However, during qPCR Taq polymerase degrades the probe releasing the fluorophore which is then allowed to fluoresce. The amount of fluorescence is therefore directly proportional to the amount of product formed during the course of the qPCR reaction (Livak *et al.*, 1995). All qPCR reactions were set up in 384 multiwell plates (Roche, UK). All samples and standards were run in triplicate. A mastermix was prepared for each plate (Table 2.6).The master mix was vortexed and centrifuged. 10 µl of mastermix was then added to 5 µl cDNA sample in a 384 well plate format. A different mastermix was required for each gene of interest/ primer and probe set. Each 384 well plate was then sealed, spun down and run on a standard probe run protocol on the Lightcycler 480 and software version 1.5 (Roche, UK).

Table 2.6 Probe Mastermix. (Lightcycler® 480 probes master mix was supplied by Roche, UK, primer and probe sets were obtained from MWG, UK)

Reagents	Volume
Lightcycler® 480 probes master mix	7.5 µl
Forward Primer	0.45 µl
Reverse Primer	0.45 µl
Probe	0.3 µl
ddH_20 (Sigma, UK)	1.3 µl
Total volume	10 µl

2.8.6.1.1 Design of Primers and probes

Primer and probe sets were required for MMP9, GZMB, ECGF1 and EDNRA. Gene sequences for these genes were found using Ensemble database http://www.ensembl.org/index.html accession numbers can be found in Table 2.7 along with primer and probe sequences. Primer and probe sets were designed using Primer Express™ software version 1.5 (Applied Biosystems, UK). mRNA expression data was normalised via the use of the housekeeping gene 36B4. Housekeeping genes are transcribed at a relatively constant level in several species and across many or all known conditions. The housekeeping gene's products are typically needed for maintenance of the cell. Their expression is unaffected by experimental conditions.

Table 2.7 Primer and probe sequences.

Gene	Accession number	Forward Primer (5'→3')	Reverse Primer (5'→3')	Probe (5'→3')
MMP9	ENSRNOG00000017539	TGGCACCATCATAACATCACCT TM = 58, GC= 46%	GAGTCATCGATCACGTCTCGC TM = 61, GC= 57%	TGGATCCAAAGCTACACCGAAGACTTGC TM = 66, GC= 50%
GZMB	ENSRNOG00000032868	TGTGCTGACTGCTGCTCACTG TM = 61, GC= 57%	TGTTCTTTGATGTTGTGGGCC TM = 58, GC= 48%	TCGGGAAGCAAAATAAATGTCACGTTGG TM = 64, GC= 43%
ECGF1	ENSRNOG00000032394	CTGCGGGACCTGGTCATT TM = 58, GC= 61%	GGTCTTGGGTTTCTGCCTGTC TM = 61, GC= 57%	CTAGGAGGCGCCATTCTTTGGCTTAGC TM = 68, GC= 56%
EDNRA	ENSRNOG00000012721	TTGCCCTCAGCGAACACC TM = 58, GC= 61%	CAACCAAGCAGAAGACGGTCT TM = 60, GC= 52%	CAAGCAGCGTCGAGAGGTGGCA TM = 66, GC= 64%
IGF2	ENSRNOG00000020369	CAAGCGGACCCGACCT TM = 59, GC= 75%	TGAGAAGCACCAACATCGACTT TM = 58, GC= 45%	CGGCCTTGCGGTACCAATGGG TM = 69, GC= 65%
36B4	-	GCTTCATTGTGGGAGCAGACA TM = 57, GC= 52%	CATGGTGTTCTTGCCCATCAG TM = 56, GC= 52%	TCCAAGCAGATGCAGCAGATCCGC TM = 64, GC= 58%

2.9 Power Calculations

Statistical power was assessed if the hypothesis was rejected due to a non-significant difference and the test group differed from the control group by 25% or more. Power calculations were performed using the online researcher's toolkit sample size calculator http://www.dssresearch.com/toolkit/spcalc/power_a2.asp (DSS Research). Means and standard deviations were calculated using SPSS (version

14.0). The probability of a type I error (α) was set at 0.05 and of a type II error (β) set at 0.10, to give a power (β-1) of 0.90.

2.10 Statistical Analysis

All data was analysed using the Statistical Package for Social Sciences (SPSS, Inc, Chicago, IL, Version 14.0). Differences between groups were assessed using a mixed model ANOVA (fixed factors, maternal diet/ mating cross, sex and age), unless otherwise indicated in the text. Values are expressed as mean ± S.E.M. $P<0.05$ was considered as significant. Analysis of data arising from fetal programming studies needs to be robust in order to avoid errors associated with within-litter effects (Osmond *et al.*, 2005; Walters and Edwards, 2003). As the present study utilised multiple pups from the same dam, litter of origin was included as a fixed nested factor in all analyses (Festing, 2006). Analyses were performed within generations, with no consideration of influences between generations, except for a comparison of blood pressure across the three generations. Within each generation the 18% casein control diet (F1), or the Con x Con breeding cross (F2 and F3) was used as the control group. In order to maintain litter of origin within the analyses *Post hoc* testing was not possible.

3.0 Maternal protein restriction during pregnancy and postnatal High Fat Feeding- body composition

3.1 Introduction

As previously discussed (section 1.7), a range of animal models have been used in order to study the basic physiological principles of the fetal programming hypothesis. The sheer variety and complexity of experimental models has been a key factor in the search for the mechanistic association between pre- and postnatal factors and their physiological impact in later life (Langley-Evans *et al.*, 1996b; Rajakumar *et al.*, 1998; Vickers *et al.*, 2000; Woodall *et al.*, 1996b). Most experimental work undertaken has concentrated on a restriction of fetal growth (Rajakumar *et al.*, 1998; Woodall *et al.*, 1996b), contrary to the fact that epidemiological studies suggest that the phenomenon of fetal programming occurs within a normal distribution of birth weights and sizes (Barker, 1994). The assumption has been that the stimuli which impair growth will simultaneously induce fetal programming (Langley-Evans, 2004). Alterations to maternal diet, particularly the feeding of a maternal low protein (MLP) diet are commonly used to elicit intrauterine growth retardation (IUGR) in a experimentally efficient and reproducible way (Langley-Evans, 2000, 2001; Langley-Evans *et al.*, 1996b). It is however important to note that offspring of animals exposed to a 9% casein MLP diet during gestation are of similar birthweights to controls (Langley-Evans *et al.*, 1996b) emphasising the complexity of the issue.

The feeding of a low protein diet during rat gestation has been shown to have an extensive effect on fetal development (Langley-Evans, 2001; Langley-Evans *et al.*, 2003). The postnatal outcomes for offspring exposed to MLP diet during gestation have been widely studied (section 1.8) and the timing of LP intervention

has proved to be an essential determinant of programmed effects (Langley-Evans *et al.*, 1996b; Langley-Evans and Nwagwu, 1998).

Previous work on the MLP diet has demonstrated that MLP exposed offspring of the F1 generation consume significantly less than controls when fed a chow diet postnatally. However they have greater energy intake when placed on self selection diets (Bellinger and Langley-Evans., 2005). Indeed previous studies have indicated that MLP exposed animals exhibit altered appetite preferences, with a preference for higher fat, more energy dense food types (Bellinger *et al.*, 2004). HF diets have been routinely used by several research groups in order to induce obesity following maternal dietary change (Ortmann *et al.*, 2003; Ozanne *et al.*, 2004; Vickers *et al.*, 2000). Previously, Vickers and co-workers (2000) reported hypertension, hyperphagia and obesity in F1 offspring following severe maternal nutrient restriction (70% of *ad libitum* food intake) throughout gestation. Vickers *et al.*, (2000) noted differences in feeding behaviour and weight gain between adequately nourished and undernourished groups of offspring (Vickers *et al.*, 2000). It was noted that weight gain and adiposity could be amplified by the manipulation of the postnatal diet, and that symptoms associated with the development of the metabolic syndrome were greatest in animals that were both nutrient restricted *in utero* and then fed a hypercaloric or high fat diet postnatally (Vickers *et al.*, 2000). Bellinger and colleagues noted that F1 MLP-exposed animals were hypophagic, however, when offered a self-selection diet they consumed more energy than control offspring. This suggested that the animals had a hyperphagic tendency when offered an energy dense diet (Bellinger, 2005; Bellinger *et al.*, 2004) which is similar to the observations noted by Vickers and colleagues (2000). Very little evidence exists with relation to food intake and preferences in MLP restricted animals of the F2 and F3

generations, Work by Zambrano and colleagues (2005) has indicated that there is no difference in food intake within the F2 generation (Zambrano *et al*., 2005).

Body composition of the F1 generation following maternal protein restriction is well documented. Previous studies have indicated that adult body composition does not appear to differ between MLP exposed animals and control animals, although there is some evidence that MLP exposed male rats deposit more gonadal fat with aging (Bellinger *et al*., 2004). Currently no data exists on the body composition of the F2 and F3 generations following maternal protein restriction during gestation.

The work described within this chapter aimed to explore the potential impact of a postnatal high fat (HF) diet (Western diet), against the background of prenatal undernutrition. Dietary challenges in postnatal life have been used previously to show their importance in the manifestation of programmed disease and obesity states (Bellinger *et al.*, 2004; Breier *et al.*, 2001; Jones, 1983; Vickers *et al.*, 2000). Similar approaches were used in this study, as increased food intake may be important in the development of obesity and onset of adult diseases such as CVD and type II diabetes. It was hypothesised that a more palatable energy dense diet would unmask any programmed hyperphagia in the MLP crosses of the F2 and F3 generations.

3.2 Objectives

Previous literature in this area has concentrated on the physiological and metabolic profile of MLP-exposed F1 generation offspring, fed a high fat diet postnatally. This study seeks to explore the effects the prenatal protein insult has for

further generations (F2 & F3), utilising a well-established and characterised animal model of nutritional programming. The main aims of this chapter were to:

- Assess, using a variety of techniques, possible changes to body composition of the F2 and F3 generations brought about by the exposure to a MLP diet in the F0 generation.
- Utilise a postnatal HF diet to expose programmed hyperphagia in MLP crosses of the F2 and F3 generations.
- Use a variety of physiological, biochemical and analytical techniques to determine possible changes to body composition of the F2 and F3 generations brought about by the programmed hyperphagia.

3.3 Materials and methods

3.3.1 Animal Procedures

Virgin Wistar rats were mated and randomly allocated to be fed either a control diet (n=10) or a LP diet (n=10) (as described in section 2.1.1) throughout gestation. At birth, dams were returned to standard laboratory chow (section 2.1.1) and resulting offspring were culled to 8 animals per litter (section 2.1.2). Upon weaning (as described in section 2.1.2), rats were randomly allocated to either HF (section 2.1.5) or standard chow diet (Table 2.2) until the time of cull (section 2.3). F2 and F3 generations were produced as described in sections 2.1.3 & 2.1.4 and once again randomly allocated to be fed either HF or standard chow diet postnatally. F2 and F3 generation offspring were also culled at 10 weeks of age (section 2.3).

3.3.2 Food Intake and weight measurements

Food intake was monitored in all animals for a period of 3 consecutive days at 5, 7 and 9 weeks of age, animals were singly housed during this time (section 2.1.5). Animals were also weighed at weekly intervals until the time of cull (10 weeks).

3.3.3 Biochemical endpoints

Glucose concentrations in plasma from non-fasted rats were analysed using the Trinder method of enzymatic colorimetric analysis (section 2.4.3.2). Plasma triglyceride and cholesterol concentrations were analysed using a commercially available kit (section 2.4.3.1) and non-fasted insulin concentrations were analysed using a commercial ELISA kit (section 2.4.3.3).

3.3.4 Statistical analysis

All data is presented as mean ± SEM, and was analysed using the Statistical Package for Social Sciences (SPSS) version 14. Differences between groups were assessed using a mixed model ANOVA (fixed factors, maternal diet, sex, diet and age), unless otherwise indicated, $P<0.05$ was considered as significant.

3.4 Birth Outcomes

The number of successful pregnancies, litter size, birth weight and the ratio of male to female offspring was unaffected by maternal diet or mating cross in all 3 generations when compared to Con animals (data not shown). Average litter size was fourteen, with a ratio of seven males to seven female offspring.

3.4.1 Growth Curves

Figure 3.1 Growth Curve of the F1 generation.

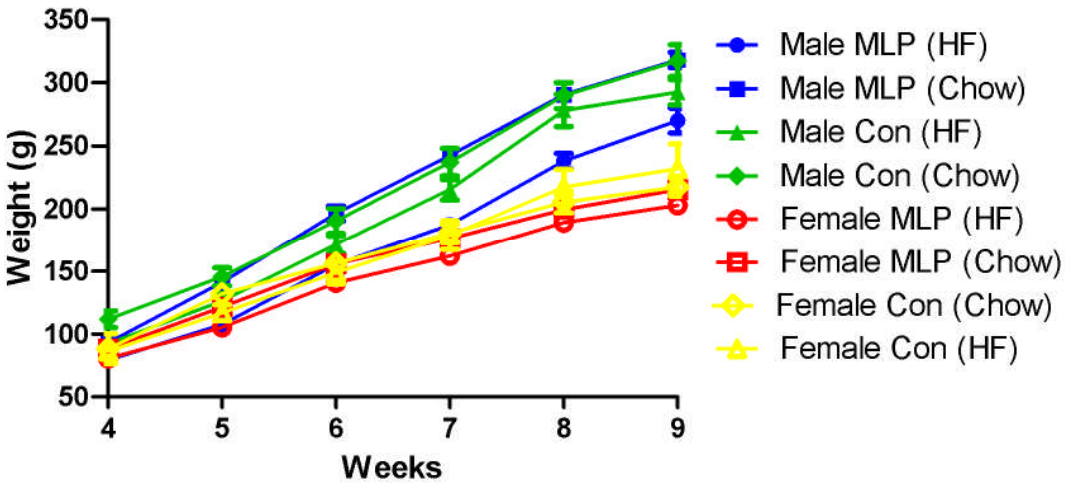


Figure 3.1 Data is shown as mean ± SEM, for Control diet male chow (n=10 male HF (n=10); female chow (n=10 female HF (n=9). MLP diet male chow (n=8); male HF (n=10); female chow (n=9) and female HF (n=11).

Figure 3.2 Growth curves of the F2 generation.

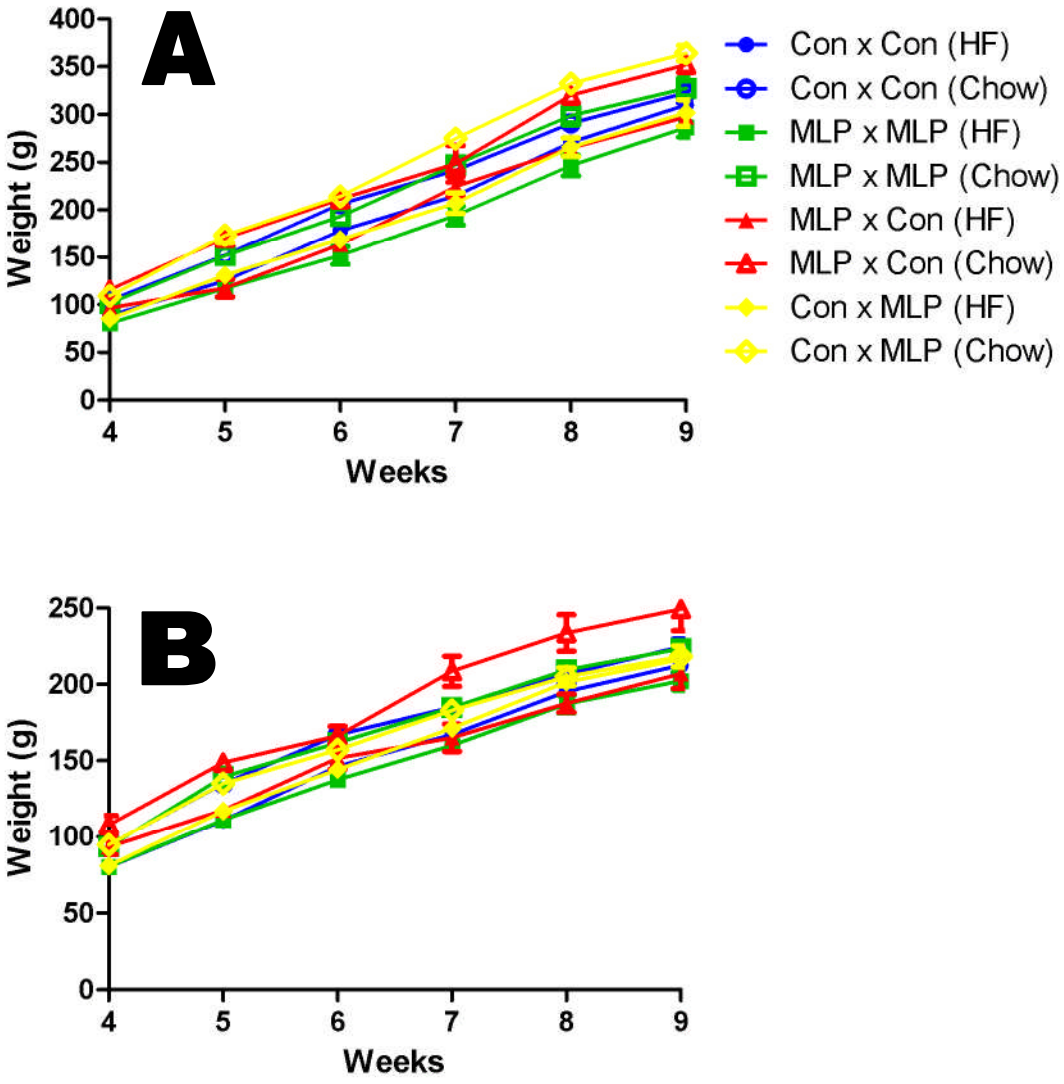


Figure 3.2 Data is shown as mean ± SEM, Graph A represents F2 generation male growth curve. For Con x Con chow (n=11); Con x Con HF (n=11); MLP x MLP chow (n=13); MLP x MLP HF (n=10); MLP x Con chow (n=6); MLP x Con HF (n=7) Con x MLP chow (n=8) and Con x MLP HF (n=8). Graph B represents F2 generation female growth curve. For Con x Con chow (n=11); Con x Con HF (n=11); MLP x MLP chow (n=7); MLP x MLP HF (n=11); MLP x Con chow (n=10); MLP x Con HF (n=8) Con x MLP chow (n=8) and Con x MLP HF (n=8).

Figure 3.3 Growth curve of the F3 generation.

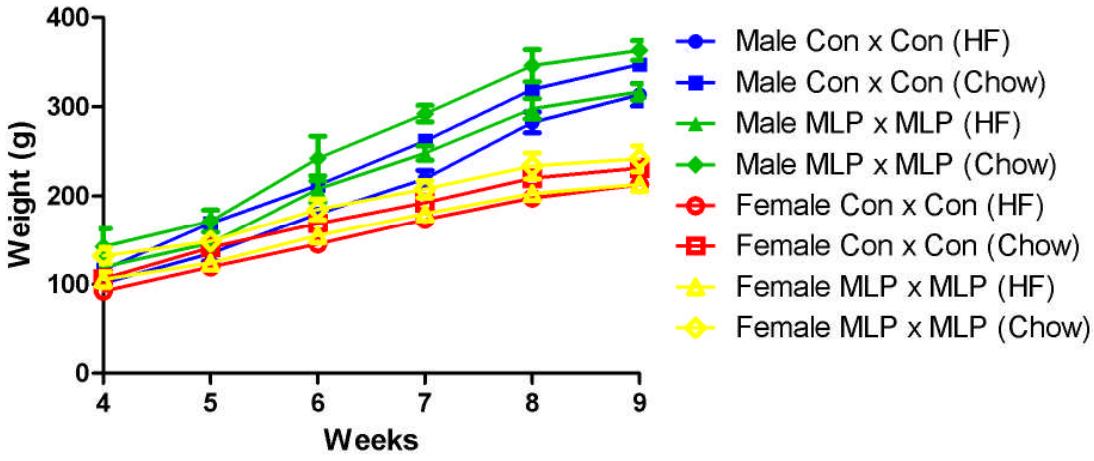


Figure 3.3 Data is shown as mean ± SEM. For male Con x Con chow (n=11); Con x Con HF (n=11); MLP x MLP chow (n=7) MLP x MLP HF (n=8); female Con x Con chow (n=9); Con x Con HF (n=9); MLP x MLP chow (n=8) and MLP x MLP HF (n=8).

3.5 Food Intake

3.5.1 F1 generation

Table 3.1 shows food intake (grams per day per kilogram body weight) of the F1 generation offspring at 5, 7 and 9 weeks of age. Food intake was similar in animals exposed to both control and MLP diets *in utero* at all time points monitored. In both maternal dietary groups, food intake was similar in males and females at 5, 7 and 9 weeks of age. When maintained on a HF diet, 5 week-old animals, irrespective of sex, substantially lowered their food intake by approximately (two-fold) 50% ($P<0.001$) compared to chow fed animals. This trend continued at 7 ($P<0.001$) and 9 ($P<0.001$) weeks of age although the difference in food intake did become less pronounced.

Table 3.3 shows energy intake (MJ per day per kilogram body weight) in the F1 generation offspring at 5, 7 and 9 weeks of age. Energy intakes were similar in

both maternal dietary groups at 5, 7 and 9 weeks of age. Energy intakes were also similar in males and females at all time points monitored. When fed the HF diet, both male and female animals initially lowered their energy intakes ($P<0.05$) by approximately 10% at 5 weeks of age. However, by 7 weeks of age energy intakes had returned to values comparable to that of animals fed a chow diet, at 9 weeks of age no difference in energy intake between postnatal dietary groups was noted.

3.5.2 F2 generation

Table 3.2 shows food intake (grams per day per kilogram body weight) of the F2 generation offspring at 5, 7 and 9 weeks of age. Food intake at 5 weeks of age in the F2 generation was influenced by an interaction of mating cross and postnatal diet ($P<0.05$). Food intake was greater ($P<0.05$) in the MLP x Con and MLP x MLP mating cross male animals maintained on a chow diet, compared to controls (Con x Con), However female animals exhibited a decrease in food intake in all MLP mating crosses compared to controls. The feeding of the HF diet substantially increased ($P<0.05$) food intake in the MLP x Con mating cross in both male and female animals relative to all other mating crosses. At 7 weeks of age food intake patterns were similar to that seen in 5 week old animals. Food intakes of male and female animals fed a chow postnatal diet were similar to controls (Con x Con) in all mating crosses. When fed the HF diet there was a marked increase ($P<0.05$) in food intake (approximately 10%) in the MLP x Con mating cross compared to all other mating crosses, in both male and female animals. At 9 weeks of age food intake was similar among all mating crosses. Intake was also similar in male and female animals. The feeding of a HF diet substantially reduced ($P<0.001$) grams intake per

day per kilogram bodyweight in both male and female animals, compared to animals fed a postnatal chow diet.

Table 3.4 shows the energy intakes (MJ per day per kilogram body weight) in the F2 generation offspring at 5, 7 and 9 weeks of age. Energy intake at 5 weeks of age was similar among male and female animals fed a chow diet irrespective of mating cross. However, when fed the postnatal HF diet, energy intakes were reduced ($P<0.05$) by approximately 20% in both male and female animals in all groups, except the MLP x Con mating cross. At 7 weeks of age the trend was similar to that seen at 5 weeks. Energy intakes were similar among male and female animals fed a postnatal chow diet irrespective of mating cross. However, when fed the postnatal HF diet energy intakes lowered ($P<0.05$). The decrease seen at 7 weeks of age was less severe (approximately 10%) than was noted previously at 5 weeks of age. At 9 weeks of age, energy intakes were similar among all mating crosses irrespective of sex and postnatal diet.

3.5.3 F3 generation

Table 3.5 shows food intake in the F3 generation offspring at 5, 7 and 9 weeks of age. At 5 weeks of age food intakes were similar in all animals regardless of sex and mating cross when fed the postnatal chow diet. However, when fed the postnatal HF diet, intakes were lower ($P<0.05$, approximately 35%) compared to animals fed the postnatal chow diet. This decrease continued at 7 weeks of age, with both male and female animals from the Con x Con mating cross exhibiting a lower food intake of around 35%. However, the lower intake in the MLP x MLP mating cross by this time had generally become less severe (approximately 25%). No difference in intake related to mating cross or sex, was noted in 7-week-old animals

fed on a postnatal chow diet. At 9 weeks of age, food intakes were similar in both male and female animals irrespective of mating cross. Postnatal HF feeding lowered ($P<0.001$) food intake in male and female animals by approximately 40% in both Con x Con and MLP x MLP groups.

Table 3.6 shows energy intake (MJ per day per kilogram body weight) in the F3 generation offspring at 5, 7 and 9 weeks of age. Energy intake at 5 weeks of age was similar among both male and female animals regardless of mating cross and postnatal diet. By 7 weeks of age energy intakes had reduced from values attained at 5 weeks. Energy intake was similar in both male and female animals and was unaffected by mating cross and postnatal diet. At 9 weeks of age energy intakes remained unaffected by mating cross. Postnatal HF feeding reduced ($P<0.05$) energy intake in both male and female animals.

Table 3.1 F1 generation food intake at 5, 7 and 9 weeks of age.

Generation	Maternal diet/cross	Postnatal diet		Males				Females		
			n	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F1	Control	Chow	11	183.2±11.4	129.9±9.1	107.6±11.7	9	180.7±6.9	130.3±4.0	117.9±8.8
	Control	HF	10	105.6±3.5	81.4±2.2	64.3±3.0	10	116.1±10.2	101.1±11.0	82.9±5.5
	MLP	Chow	8	195.9±11.9	132.9±6.2	98.4±2.9	9	202.7±13.6	145.4±7.4	118.7±4.9
	MLP	HF	10	101.3±8.2	91.5±9.4	81.5±12.2	11	110.6±7.8	81.1±2.9	76.9±6.2
P for effect of maternal diet (F1)				NS	NS	NS	-	NS	NS	NS
P for effect of postnatal diet (F1)				P<0.001	P<0.001	P<0.001	-	P<0.001	P<0.001	P<0.001
P for effect of sex (F1)				NS	NS	NS	-	NS	NS	NS
P for interaction of maternal x postnatal diets (F1)				NS	NS	NS	-	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as grams per day per kilogram body weight. NS= not significant.

Table 3.2 F2 generation food intake at 5, 7 and 9 weeks of age.

Generation	Maternal diet/cross	Postnatal diet		Males				Females		
			n	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F2	Con x Con	Chow	11	175.8±7.8	118.4±5.4	81.0±8.3	11	204.9±37.3	121.5±4.9	89.5±10.9
	Con x Con	HF	11	97.3±1.8	72.4±3.2	74.5±15.1	11	101.9±7.7	77.6±4.3	63.5±2.1
	Con x MLP	Chow	8	171.2±14.2	123.1±7.3	98.9±2.9	8	169.8±22.9	121.9±8.5	118.8±4.6
	Con x MLP	HF	8	94.6±5.9	82.0±5.4	68.7±14.2	8	91.9±7.1	78.7±5.7	67.6±3.9
	MLP x Con	Chow	6	182.5±14.6	132.8±11.9	97.4±6.3	10	195.8±40.4	118.7±4.8	112.5±6.8
	MLP x Con	HF	7	120.9±12.3	87.4±10.3	55.6±3.2	8	129.5±30.7	87.3±7.4	59.7±3.1
	MLP x MLP	Chow	13	194.9±7.0	122.6±4.1	89.6±1.8	7	197.3±9.4	118.3±6.9	100.4±4.8
	MLP x MLP	HF	10	91.2±7.2	80.7±6.7	59.8±2.5	11	79.1±6.4	73.7±2.6	70.2±5.6
P for effect of mating cross (F2)				P<0.05	P<0.05	NS	-	P<0.05	P<0.05	NS
P for effect of postnatal diet (F2)				P<0.001	P<0.001	P<0.001	-	P<0.001	P<0.001	P<0.001
P for interaction of mating cross x postnatal diet (F2)				P<0.05	P<0.05	NS	-	P<0.05	P<0.05	NS
P for effect of sex (F2)				P<0.05	NS	NS	-	P<0.05	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as grams per day per kilogram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

Table 3.3 F1 generation energy intake at 5, 7 and 9 weeks of age.

Generation	Maternal diet/cross	Postnatal diet		Males				Females		
			n	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F1	Control	Chow	11	3.0±0.2	2.1±0.1	1.8±0.2	9	3.0±0.1	2.1±0.1	1.9±0.1
	Control	HF	10	2.7±0.1	2.0±0.1	1.6±0.1	10	2.9±0.3	2.5±0.3	2.1±0.1
	MLP	Chow	8	3.2±0.2	2.2±0.1	1.6±0.1	9	3.3±0.2	2.4±0.1	2.0±0.1
	MLP	HF	10	2.5±0.2	2.3±0.2	2.1±0.3	11	2.8±0.2	2.0±0.1	1.9±0.2
P for effect of maternal diet (F1)				NS	NS	NS	-	NS	NS	NS
P for effect of postnatal diet (F1)				P<0.05	NS	NS	-	P<0.05	NS	NS
P for effect of sex (F1)				NS	NS	NS	-	NS	NS	NS
P for interaction of maternal x postnatal diets (F1)				NS	NS	NS	-	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as MJ per day per kilogram body weight. NS= not significant.

Table 3.4 F2 generation energy intake at 5, 7 and 9 weeks of age.

Generation	Maternal diet/cross	Postnatal diet		Males				Females		
			n	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F2	Con x Con	Chow	11	2.9±0.1	1.9±0.1	1.3±0.1	11	3.4±0.6	2.0±0.1	1.5±0.2
	Con x Con	HF	11	2.4±0.0	1.8±0.1	1.9±0.4	11	2.6±0.2	1.9±0.1	1.6±0.1
	Con x MLP	Chow	8	2.8±0.2	2.0±0.1	1.6±0.1	8	2.8±0.4	2.0±0.1	2.0±0.1
	Con x MLP	HF	8	2.4±0.1	2.1±0.1	1.7±0.4	8	2.3±0.2	2.0±0.1	1.7±0.1
	MLP x Con	Chow	6	3.0±0.2	2.2±0.2	1.6±0.1	10	3.2±0.7	1.9±0.1	1.8±0.1
	MLP x Con	HF	7	3.0±0.3	2.2±0.3	1.4±0.1	8	3.3±0.8	2.2±0.2	1.5±0.1
	MLP x MLP	Chow	13	3.2±0.1	2.0±0.1	1.5±0.0	7	3.2±0.2	2.4±0.1	1.7±0.1
	MLP x MLP	HF	10	2.3±0.2	2.0±0.2	1.5±0.1	11	2.0±0.2	1.9±0.1	1.8±0.1
P for effect of mating cross (F2)				NS	NS	NS	-	NS	NS	NS
P for effect of postnatal diet (F2)				P<0.05	NS	NS	-	P<0.05	NS	NS
P for interaction of F2 or F3 mating cross x postnatal diet (F2)				NS	NS	NS	-	NS	NS	NS
P for effect of sex (F2)				NS	NS	NS	-	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as MJ per day per kilogram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

Table 3.5 F3 generation food intake at 5, 7 and 9 weeks of age.

Generation	Maternal diet/cross	Postnatal diet		Males				Females		
			n	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F3	Con x Con	Chow	11	167.5±13.2	123.5±5.4	109.1±5.8	9	162.2±13.9	128.9±4.1	121.2±5.9
	Con x Con	HF	11	97.2±15.3	74.9±3.3	60.6±3.5	9	87.5±6.0	74.3±4.9	63.7±1.9
	MLP x MLP	Chow	7	172.9±7.6	102.2±4.3	103.7±3.4	8	146.8±13.1	116.6±5.1	122.8±4.4
	MLP x MLP	HF	8	115.1±15.3	74.7±4.6	60.4±4.6	8	103.2±17.6	69.8±4.1	66.3±3.9
P for effect of mating cross (F3)				NS	NS	NS	-	NS	NS	NS
P for effect of postnatal diet (F3)				P<0.05	P<0.05	P<0.001	-	P<0.05	P<0.05	P<0.001
P for interaction of mating cross x postnatal diet (F3)				NS	NS	NS	-	NS	NS	NS
P for effect of sex (F3)				P<0.05	NS	NS	-	P<0.05	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as grams per day per kilogram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant

Table 3.6 F3 generation energy intake at 5, 7 and 9 weeks of age.

Generation	Maternal diet/cross	Postnatal diet		Males				Females		
			n	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F3	Con x Con	Chow	11	2.7±0.2	2.0±0.1	1.8±0.1	9	2.7±0.2	2.1±0.1	2.0±0.1
	Con x Con	HF	11	2.4±0.4	1.9±0.1	1.5±0.1	9	2.2±0.2	1.9±0.1	1.6±0.1
	MLP x MLP	Chow	7	2.7±0.2	1.7±0.1	1.7±0.1	8	2.4±0.2	1.9±0.1	2.0±0.1
	MLP x MLP	HF	8	2.9±0.4	1.9±0.1	1.5±0.1	8	2.6±0.4	1.8±0.1	1.7±0.1
P for effect of mating cross (F3)				NS	NS	NS	-	NS	NS	NS
P for effect of postnatal diet (F3)				NS	NS	P<0.05	-	NS	NS	P<0.05
P for interaction of mating cross x postnatal diet (F3)				NS	NS	NS	-	NS	NS	NS
P for effect of sex (F3)				NS	NS	NS	-	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as MJ per day per kilogram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant

3.6 Feed efficiency

3.6.1 F1 generation

Table 3.7 shows the feed efficiency (weight gained per unit of energy intake) of the F1 generation offspring at 5, 7 and 9 weeks of age. Feed efficiency was similar in animals exposed to control and MLP diets *in utero,* at all time points. In both maternal dietary groups, efficiency in males was significantly (5 weeks $P<0.001$, 7 & 9 weeks $P<0.05$) higher than in females with approximately 20% greater weight gain at any given level of food intake. When fed the HF diet, male animals increased efficiency two-fold at 5 weeks of age ($P<0.001$). This trend continued at 7 and 9 weeks of age ($P<0.001$). Females exhibited the same response to high fat feeding, but despite this their efficiency only attained levels noted in chow fed males. Throughout the study, weight gain per unit food intake was greater with HF feeding than with chow and males had greater efficiency than females. In F1 animals the prenatal diet had no influence on feed efficiency.

3.6.2 F2 generation

Table 3.8 shows the feed efficiency of the F2 generation offspring at 5, 7 and 9 weeks of age. Feed efficiency in the F2 generation was similar in all mating crosses at 5 and 7 weeks of age, however by 9 weeks, efficiency in all MLP mating crosses was notably reduced ($P<0.05$) compared to the Con x Con group. Similar to the F1 generation, efficiency in males was significantly ($P<0.001$) higher than in females at 5 and 7 weeks of age. This trend intensified with increasing age and by 9 weeks of age, male animals from the Con x Con mating cross had approximately 40% greater weight gain per unit of food intake compared to females of the same group.

However, efficiency in all three MLP mating crosses remained similar in males and females. When fed the HF diet, male animals at 5 and 7 weeks of age, regardless of mating cross, had greater efficiency approximately two-fold ($P<0.001$). By 9 weeks of age, the feeding of the HF postnatal diet in all MLP mating crosses had greater efficiency (approximately 40%). However efficiency levels with HF feeding in the Con x Con group remained similar to values attained by chow fed animals. Females exhibited the same response to HF feeding. Despite this their efficiency was still lower than values attained by male animals. Throughout the study weight gain per unit food intake in the F2 generation was greater with HF feeding than with chow and males animals had greater efficiency than females. In F2 generation animals, mating cross had no influence on feed efficiency at 5 and 7 weeks of age, however by 9 weeks all MLP mating crosses exhibited greater efficiency compared to controls (Con x Con), regardless of sex and postnatal diet.

3.6.3 F3 generation

Table 3.9 shows feed efficiency in the F3 generation offspring at 5, 7 and 9 weeks of age. At 5 and 7 weeks of age feed efficiency was similar regardless of mating cross. By 9 weeks of age efficiency in animals from the MLP x MLP mating cross was significantly reduced ($P<0.05$) compared to controls. This was, however dependent upon postnatal diet. Efficiency in males was significantly ($P<0.05$) higher than in females at 7 and 9 weeks of age, following the trend outlined in the F1 and F2 generations. There was no difference between sexes at 5 weeks. When fed a postnatal HF diet, male animals regardless of mating cross, elevated their efficiency approximately two-fold (7 weeks $P<0.05$ & 9 weeks $P<0.001$), another trait that was noted in previous generations. Females of the F3 generation exhibited the same

response to high fat feeding. However, their efficiency remained lower than their male counterparts. Feed efficiency of the F3 generation was greater with HF feeding than with chow diet. Male animals had greater efficiency than females. In the F3 generation animals, mating cross had no influence on feed efficiency at 5 and 7 weeks of age, however by 9 weeks the MLP x MLP mating cross exhibited reduced efficiency compared to controls. An interaction of mating cross and postnatal diet was noted ($P<0.05$).

Table 3.7 F1 generation feed efficiency at 5, 7 & 9 weeks of age.

Generation				Males				Females		
	Maternal diet/cross	Postnatal diet	*n*	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F1	Control	Chow	11	25±2	22±2	14±2	9	16±2	12±2	6±1
	Control	HF	10	51±3	41±3	34±3	10	32±2	24±2	23±4
	MLP	Chow	8	24±3	19±2	17±5	9	17±1	11±1	9±1
	MLP	HF	10	47±3	36±3	29±3	11	37±3	29±4	19±2
P for effect of maternal diet (F1)				NS	NS	NS	-	NS	NS	NS
P for effect of postnatal diet (F1)				P<0.001	P<0.001	P<0.001	-	P<0.001	P<0.001	P<0.001
P for effect of sex (F1)				P<0.001	P<0.05	P<0.05	-	P<0.001	P<0.05	P<0.05
P for interaction of maternal x postnatal diets (F1)				NS	NS	NS	-	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as weight gained per unit food intake (%).NS= not significant.

Table 3.8 F2 generation feed efficiency at 5, 7 & 9 weeks of age.

Generation				Males				Females		
	Maternal diet/cross	Postnatal diet	*n*	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F2	Con x Con	Chow	11	27±1	24±2	35±14	11	23±1	18±2	19±3
	Con x Con	HF	11	55±2	49±3	40±4	11	47±3	30±2	26±2
	Con x MLP	Chow	8	26±3	28±2	13±1	8	20±4	15±3	11±2
	Con x MLP	HF	8	52±4	57±4	35±5	8	47±5	34±5	24±4
	MLP x Con	Chow	6	32±7	29±0	12±2	10	19±3	20±2	12±3
	MLP x Con	HF	7	44±5	43±6	25±2	8	43±5	43±4	16±5
	MLP x MLP	Chow	13	26±2	18±2	18±2	7	20±2	11±2	16±4
	MLP x MLP	HF	10	60±4	46±5	34±1	11	57±11	30±3	16±4
P for effect of mating cross (F2)				NS	NS	P<0.05	-	NS	NS	P<0.05
P for effect of postnatal diet (F2)				P<0.001	P<0.001	P<0.001	-	P<0.001	P<0.001	P<0.001
P for effect of sex (F2)				P<0.05	P<0.001	P<0.001	-	P<0.05	P<0.001	P<0.001
P for interaction of mating cross x postnatal diet (F2)				NS	NS	P<0.05	-	NS	NS	P<0.05

Data shows mean ± SEM for *n* observations per group. Data are represented as weight gained per unit food intake (%). For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant

Table 3.9 F3 generation feed efficiency at 5, 7 & 9 weeks of age.

Generation				Males				Females		
	Maternal diet/cross	Postnatal diet	*n*	Week 5	Week 7	Week 9	*n*	Week 5	Week 7	Week 9
F3	Con x Con	Chow	11	28±2	19±3	16±2	9	25±3	9±2	11±2
	Con x Con	HF	11	65±9	43±2	31±2	9	49±5	21±4	20±6
	MLP x MLP	Chow	7	28±1	18±1	9±2	8	23±3	14±3	9±1
	MLP x MLP	HF	8	46±4	42±4	30±4	8	30±2	24±4	21±4
P for effect of mating cross (F3)				NS	NS	P<0.05	-	NS	NS	P<0.05
P for effect of postnatal diet (F3)				NS	P<0.05	P<0.001	-	NS	P<0.05	P<0.001
P for effect of sex (F3)				NS	P<0.05	P<0.05	-	NS	P<0.05	P<0.05
P for interaction of mating cross x postnatal diet (F3)				NS	NS	P<0.05	-	NS	NS	P<0.05

Data shows mean ± SEM for *n* observations per group. Data are represented as weight gained per unit food intake (%). For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant

3.7 Body composition 10 weeks

3.7.1 F1 generation

Table 3.10 shows the body composition of F1 generation male animals, when culled at 10 weeks of age. Body weight at time of cull was similar in animals exposed to control and MLP diets *in utero.* All organs and fat pads were of similar size relative to body weight in chow fed male animals, irrespective of maternal dietary exposure. The feeding of a HF diet postnatally had no effect on body weight on males from the F1 generation (Table 3.10), however, liver and lung size relative to bodyweight were greater ($P<0.05$) compared to chow fed rats, in both maternal dietary groups, as were perirenal and gonadal fat (Figure 3.4) deposits ($P<0.05$). The size of all other organs relative to body weight was similar irrespective of postnatal diet.

Table 3.11 shows the body composition of F1 generation females at 10 weeks of age. As noted in male animals, body weight at time of cull was unchanged by MLP feeding and all organs and fat pads were of similar size relative to body weight in chow fed female animals. Postnatal HF feeding in the F1 generation female animals resulted in greater ($P<0.05$) liver size relative to body weight by approximately 5-10%. Lung size was similarly affected showing an increase ($P<0.05$) of approximately 7-15%. Gonadal and perirenal fat pads (Figure 3.4) were also substantially greater ($P<0.05$) by approximately 40%, on postnatal HF feeding.

Tables 3.10 and 3.11 show that body weight and organ size relative to body weight in both male and female animals of the F1 generation at 10 weeks of age were unaffected by administration of control and MLP diets *in utero.* However, postnatal

HF feeding did impact upon liver, lung, gonadal fat and perirenal fat deposition, substantially increasing organ/fat pad size relative to body weight.

Table 3.10 Male F1 generation body composition 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Body wt (g)	Organ weight/body weight (%)									
					Liver	Left kidney	Right kidney	Heart	Lung	Brain	Thymus	Spleen	Gonadal fat pad	Perirenal fat pad
F1	Control	Chow	11	383.7± 19.82	4.26± 0.20	0.37± 0.01	0.38± 0.02	0.38± 0.02	0.59± 0.03	0.47± 0.02	0.14± 0.01	0.23± 0.01	0.76± 0.07	0.71± 0.12
	Control	HF	10	381.7± 13.57	4.56± 0.18	0.39± 0.02	0.40± 0.02	0.36± 0.01	0.82± 0.09	0.47± 0.02	0.16± 0.02	0.22± 0.03	0.98± 0.1	0.97± 0.09
	MLP	Chow	8	380.8± 6.93	4.69± 0.14	0.39± 0.01	0.40± 0.01	0.39± 0.01	0.60± 0.04	0.46± 0.03	0.14± 0.01	0.22± 0.01	0.74± 0.04	0.66± 0.07
	MLP	HF	10	356.9± 15.64	4.93± 0.19	0.40± 0.01	0.40± 0.01	0.37± 0.01	0.78± 0.05	0.48± 0.03	0.17± 0.02	0.27± 0.02	0.88± 0.11	0.87± 0.13
P for effect of maternal diet (F1)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F1)				NS	P<0.05	NS	NS	NS	P<0.05	NS	NS	NS	P<0.05	P<0.05
P for interaction of maternal x postnatal diets (F1)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data represented as grams per 100 gram body weight. NS= not significant.

Table 3.11 Female F1 generation body composition 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Body wt (g)	Organ weight/body weight (%)									
					Liver	Left kidney	Right kidney	Heart	Lung	Brain	Thymus	Spleen	Gonadal fat pad	Perirenal fat pad
F1	Control	Chow	9	246.9±8.37	4.30± 0.10	0.39± 0.01	0.41± 0.01	0.43± 0.01	0.71± 0.06	0.63± 0.02	0.19± 0.01	0.25± 0.00	0.81± 0.08	0.58± 0.06
	Control	HF	10	281.9 ±23.41	4.49± 0.19	0.41± 0.01	0.42± 0.01	0.38± 0.01	0.76± 0.05	0.63± 0.04	0.17± 0.02	0.27± 0.02	1.13± 0.10	0.84± 0.11
	MLP	Chow	9	243.8±4.56	4.22± 0.08	0.40± 0.01	0.41± 0.01	0.41± 0.01	0.69± 0.02	0.69± 0.02	0.20± 0.01	0.26± 0.02	0.74± 0.13	0.47± 0.05
	MLP	HF	11	239.6 ±5.84	4.60± 0.10	0.43± 0.01	0.45± 0.01	0.44± 0.01	0.83± 0.04	0.67± 0.03	0.19± 0.01	0.33± 0.04	1.16± 0.08	0.89± 0.08
P for effect of maternal diet (F1)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F1)				NS	P<0.05	NS	NS	NS	P<0.05	NS	NS	NS	P<0.05	P<0.05
P for interaction of maternal x postnatal diets (F1)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data represented as grams per 100 gram body weight. NS= not significant.

Figure 3.4 Total abdominal Fat deposition in F1 generation

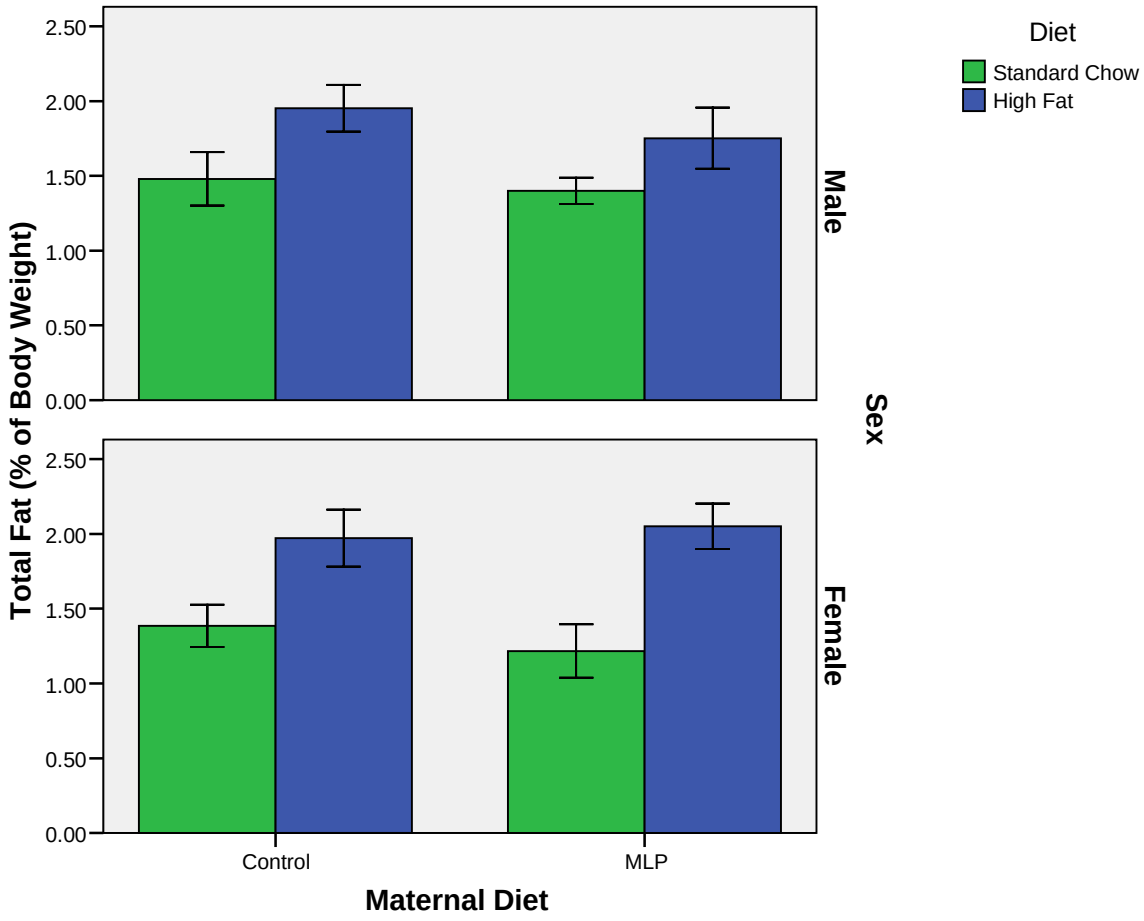


Figure 3.4 Total fat represents combined gonadal and perirenal fat deposits. Data is shown as mean ± SEM, for Control diet male chow (n=11); male HF (n=10); female chow (n=9) and female HF (n=10). MLP diet male chow (n=8); male HF (n=10); female chow (n=9) and female HF (n=11). Significant effects of postnatal diet ($P<0.05$) were noted, Data analysed using a mixed model ANOVA.

3.7.2 F2 generation

Table 3.12 shows body composition of F2 generation male animals when culled at 10 weeks of age. Body weight at time of cull was similar in animals from all mating crosses and all organs were of similar size relative to body weight in chow fed male animals of the F2 generation irrespective of mating cross. Abdominal fat deposition (Figure 3.5) was significantly influenced by mating cross. Both perirenal and gonadal fat deposits were smaller relative to body weight ($P<0.05$) in the MLP x MLP cross compared to Con x Con group. The other MLP mating crosses had similar amounts of fat to Con x Con group, male, chow fed animals. The feeding of the HF postnatal diet to the F2 generation resulted in greater ($P<0.001$, approximately 20%) lung size relative to body weight in all mating crosses. Brain size was also larger ($P<0.05$) by around 13%, regardless of mating cross. In general, HF feeding resulted in greater perirenal and gonadal fat deposition compared to chow fed animals irrespective of mating cross. HF feeding exaggerated the abdominal fat deposition phenotype observed in the chow fed animals. With HF feeding, all MLP mating crosses showed reduced ($P<0.001$) perirenal and gonadal fat deposition (approximately 18%) compared to the Con x Con mating cross fed a HF postnatal diet.

Table 3.13 shows body composition (organ size relative to body weight) of F2 generation female animals at 10 weeks of age. Body weight at time of cull was similar among female animals of the F2 generation, regardless of mating cross and all organs were of similar size relative to body weight in chow fed female animals. Gonadal fat deposition was lower ($P<0.05$) in the MLP x MLP and MLP x Con mating crosses by approximately 20% compared to their Con x Con counterparts. Perirenal fat pad size relative to body weight was similar among all mating crosses.

Feeding of the HF diet to the F2 generation females resulted in larger ($P<0.001$, approximately 15%) lung size relative to body weight in all groups. Brain size was also greater in most groups ($P<0.05$) by around 5%. However a decrease in brain size, was noted in the Con x MLP mating cross. As in the F2 generation male animals, HF feeding generally resulted in greater perirenal and gonadal fat deposition (Figure 3.5) compared to chow fed animals irrespective of mating cross. HF feeding exaggerated the abdominal fat deposition phenotype observed in the chow fed male and female animals, and as with the postnatal HF male animals, HF feeding reduced ($P<0.001$) perirenal and gonadal fat deposition (approximately 20%) in all MLP mating crosses compared to the Con x Con mating cross animals fed a HF postnatal diet.

Tables 3.12 and 3.13 show that body weight and organ size relative to body weight in both male and female animals of the F2 generation at 10 weeks of age was unaffected by mating cross. Abdominal fat deposition was lower in certain MLP mating crosses compared to controls. Postnatal HF feeding enhanced this effect and although abdominal fat deposition did increase in animals fed a HF diet compared to chow controls, the fat gain was attenuated in all MLP mating crosses compared to control animals (Con x Con) fed a HF postnatal diet (Figure 3.5).

Table 3.12 Male F2 generation body composition 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Body wt (g)	Organ weight/body weight (%)									
					Liver	Left kidney	Right kidney	Heart	Lung	Brain	Thymus	Spleen	Gonadal fat pad	Perirenal fat pad
F2	Con x Con	Chow	11	381.3± 12.88	4.52± 0.08	0.39± 0.01	0.41± 0.01	0.37± 0.02	0.60± 0.03	0.48± 0.02	0.15± 0.01	0.24± 0.01	0.71± 0.04	0.68± 0.07
	Con x Con	HF	11	381.6 ± 17.64	4.78± 0.08	0.38± 0.01	0.39± 0.01	0.37± 0.01	0.76± 0.02	0.49± 0.02	0.16± 0.01	0.24± 0.01	1.09± 0.08	1.17± 0.12
	Con x MLP	Chow	8	412.9± 10.22	4.80± 0.05	0.39± 0.01	0.39± 0.01	0.36± 0.02	0.59± 0.02	0.45± 0.01	0.13± 0.01	0.22± 0.01	0.70± 0.05	0.59± 0.07
	Con x MLP	HF	8	349.3 ± 14.07	4.66± 0.26	0.39± 0.01	0.40± 0.01	0.41± 0.02	0.75± 0.02	0.52± 0.03	0.16± 0.01	0.24± 0.01	0.88± 0.07	0.77± 0.08
	MLP x Con	Chow	6	383.3± 18.70	4.95± 0.18	0.39± 0.02	0.41± 0.02	0.37± 0.03	0.61± 0.04	0.47± 0.03	0.17± 0.01	0.23± 0.02	0.72± 0.07	0.74± 0.10
	MLP x Con	HF	7	350.5 ± 10.89	4.83± 0.20	0.39± 0.02	0.41± 0.02	0.37± 0.01	0.75± 0.04	0.54± 0.02	0.16± 0.01	0.26± 0.02	0.91± 0.12	0.83± 0.16
	MLP x MLP	Chow	13	391.2± 14.95	4.62± 0.09	0.39± 0.01	0.41± 0.01	0.39± 0.02	0.63± 0.03	0.47± 0.02	0.14± 0.01	0.22± 0.01	0.66± 0.04	0.59± 0.08
	MLP x MLP	HF	10	351.9 ± 10.51	4.82± 0.12	0.39± 0.01	0.41± 0.02	0.40± 0.02	0.80± 0.04	0.53± 0.02	0.14± 0.01	0.24± 0.01	0.90± 0.05	0.75± 0.10
P for effect of maternal cross (F2)				NS	NS	NS	NS	NS	NS	NS	NS	NS	P<0.05	P<0.05
P for effect of postnatal diet (F2)				NS	NS	NS	NS	NS	P<0.001	P<0.05	NS	NS	P<0.001	P<0.001
P for interaction of cross x postnatal diets (F2)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data represented as grams per 100 gram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

Table 3.13 Female F2 generation body composition 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Body wt (g)	Organ weight/body weight (%)									
					Liver	Left kidney	Right kidney	Heart	Lung	Brain	Thymus	Spleen	Gonadal fat pad	Perirenal fat pad
F2	Con x Con	Chow	11	249.5± 5.66	4.27± 0.08	0.41± 0.01	0.43± 0.01	0.40± 0.01	0.71± 0.02	0.68± 0.02	0.18± 0.01	0.27± 0.01	0.73± 0.05	0.47± 0.05
	Con x Con	HF	11	244.8± 7.02	4.60± 0.09	0.41± 0.01	0.43± 0.01	0.41± 0.01	0.93± 0.05	0.71± 0.02	0.19± 0.01	0.28± 0.01	1.14± 0.11	0.80± 0.10
	Con x MLP	Chow	8	239.2± 8.29	4.15± 0.08	0.39± 0.01	0.41± 0.01	0.42± 0.01	0.69± 0.01	0.74± 0.02	0.18± 0.01	0.25± 0.01	0.76± 0.09	0.48± 0.05
	Con x MLP	HF	8	244.8± 4.96	4.84± 0.12	0.45± 0.01	0.46± 0.01	0.42± 0.02	0.84± 0.06	0.71± 0.02	0.19± 0.01	0.28± 0.01	0.83± 0.09	0.65± 0.09
	MLP x Con	Chow	10	259.0± 17.42	4.36± 0.06	0.40± 0.01	0.41± 0.01	0.41± 0.03	0.72± 0.03	0.71± 0.03	0.20± 0.01	0.25± 0.01	0.62± 0.07	0.43± 0.05
	MLP x Con	HF	8	236.9± 4.29	4.84± 0.04	0.41± 0.01	0.43± 0.00	0.43± 0.02	0.84± 0.04	0.72± 0.02	0.19± 0.02	0.30± 0.01	0.88± 0.08	0.64± 0.10
	MLP x MLP	Chow	7	250.6± 3.86	4.26± 0.08	0.41± 0.01	0.42± 0.01	0.43± 0.01	0.79± 0.03	0.68± 0.02	0.17± 0.01	0.25± 0.01	0.58± 0.09	0.48± 0.05
	MLP x MLP	HF	11	228.3± 8.83	4.51± 0.14	0.43± 0.01	0.45± 0.01	0.44± 0.02	0.88± 0.04	0.77± 0.04	0.17± 0.01	0.28± 0.01	0.87± 0.10	0.69± 0.08
P for effect of maternal cross (F2)				NS	NS	NS	NS	NS	NS	NS	NS	NS	P<0.05	P<0.05
P for effect of postnatal diet (F2)				NS	NS	NS	NS	NS	P<0.001	P<0.05	NS	NS	P<0.001	P<0.001
P for interaction of cross x postnatal diets (F2)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data represented as grams per 100 gram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

Figure 3.5 Total abdominal fat deposition F2 generation

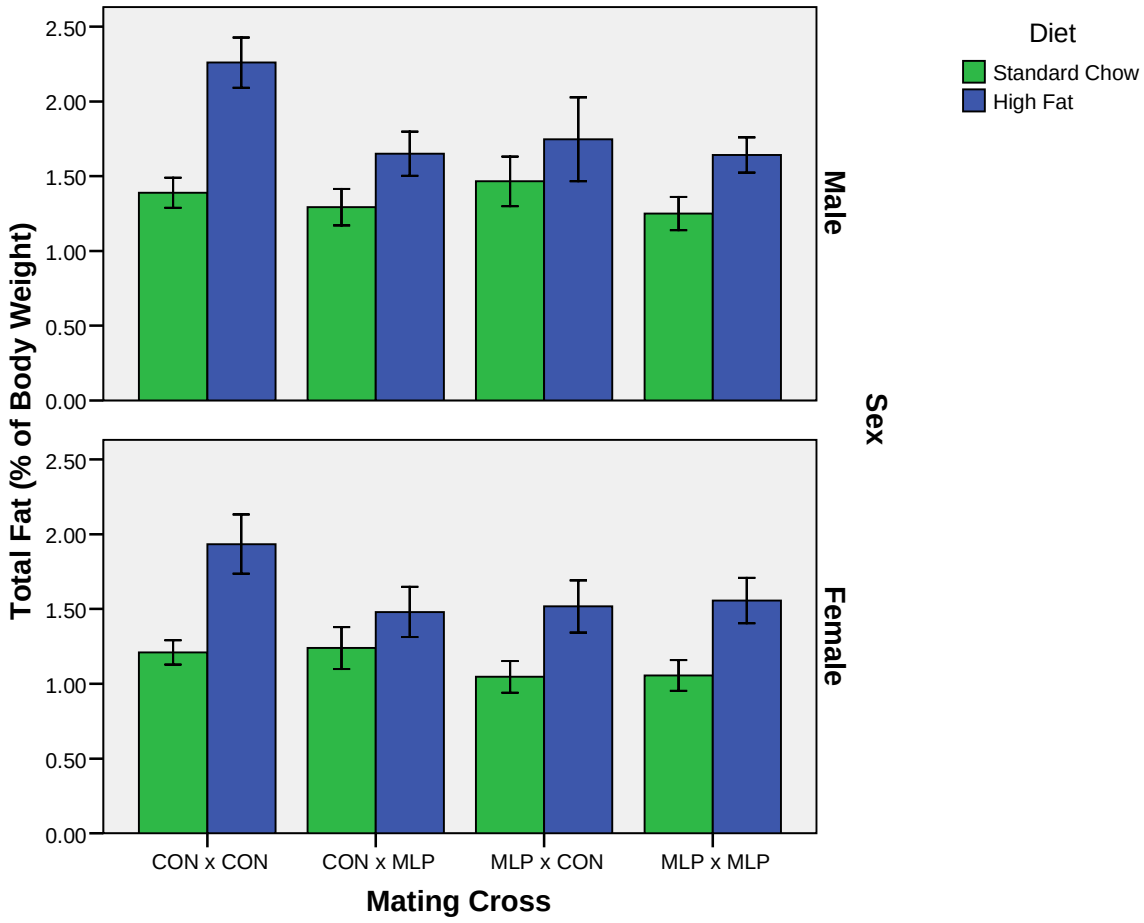


Figure 3.5 Total abdominal fat represents combined gonadal and perirenal fat deposits. Data is shown as mean ± SEM, for Con x Con male chow (n=11); male HF (n=11); female chow (n=11) and female HF (n=11), MLP x MLP male chow (n=13); male HF (n=10); female chow (n=7) and female HF (n=11), Con x MLP male chow (n=8); male HF (n=8); female chow (n=8) and female HF (n=8), MLP x Con male chow (n=6); male HF (n=7); female chow (n=10) and female HF (n=8). Significant effects of sex ($P<0.05$), diet ($P<0.001$) and cross ($P<0.05$) were noted, Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to LP diet *in utero*.

3.7.3 F3 generation

Table 3.14 shows the body composition of F3 generation male animals when culled at 10 weeks of age. Body weight at the time of cull was similar in male animals from both mating crosses and all organs and abdominal fat pads were of similar size relative to body weight in chow fed male animals. Postnatal HF feeding significantly lowered ($P<0.05$) body weight at time of cull. Liver size relative to body weight was substantially greater ($P<0.05$), and both left and right kidneys ($P<0.05$), lung ($P<0.05$) and brain ($P<0.05$) size relative to body weight were similarly affected. Abdominal fat deposition was also increased by postnatal HF feeding as both gonadal ($P<0.05$) and perirenal ($P<0.05$) fat deposition was greater (approximately 20%), irrespective of mating cross.

Table 3.15 shows body composition of F3 generation female animals at 10 weeks of age. As noted in male animals of the F3 generation, body weight at time of cull was similar in female animals regardless of mating cross. (Table 3.14) Postnatal HF feeding resulted in a smaller ($P<0.05$) body weight at the time of cull, and larger liver size relative to body weight ($P<0.05$). Both left and right kidneys ($P<0.05$), lung ($P<0.05$) and brain ($P<0.05$) size relative to body weight were also larger in HF fed animals. Gonadal ($P<0.05$) and perirenal ($P<0.05$) fat deposition was greater (approximately 33%) in animals on the HF diet compared to chow fed animals.

Tables 3.14 and 3.15 show that in the F3 generation, no differences in body weight and organ or fat pad size relative to body weight were noted between mating crosses. Postnatal HF feeding did, however, increase size of liver, kidneys, lung, brain and abdominal fat depots relative to body weight. Unlike the F2 generation, no programmed difference in fat deposition was observed in the F3 generation (Figure 3.6).

Table 3.14 Male F3 generation body composition 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Body wt (g)	Organ weight/body weight (%)									
					Liver	Left kidney	Right kidney	Heart	Lung	Brain	Thymus	Spleen	Gonadal fat pad	Perirenal fat pad
F3	Con x Con	Chow	11	401.7± 8.59	4.77± 0.12	0.37± 0.01	0.39± 0.01	0.36± 0.02	0.64± 0.03	0.46± 0.01	0.15± 0.01	0.24± 0.01	0.77± 0.03	0.65± 0.06
	Con x Con	HF	9	376.1± 14.81	4.89± 0.11	0.40± 0.02	0.42± 0.02	0.38± 0.02	0.74± 0.04	0.51± 0.02	0.17± 0.01	0.30± 0.04	1.01± 0.05	0.95± 0.09
	MLP x MLP	Chow	6	411.2± 14.21	4.35± 0.10	0.39± 0.02	0.41± 0.02	0.36± 0.01	0.65± 0.04	0.46± 0.01	0.13± 0.01	0.23± 0.02	0.68± 0.05	0.58± 0.06
	MLP x MLP	HF	8	377.8± 10.03	4.67± 0.17	0.40± 0.01	0.42± 0.01	0.38± 0.01	0.74± 0.02	0.49± 0.01	0.15± 0.02	0.25± 0.01	0.90± 0.08	0.84± 0.13
P for effect of maternal cross (F3)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F3)				P<0.05	P<0.05	P<0.05	P<0.05	NS	P<0.05	P<0.05	NS	NS	P<0.05	P<0.05
P for interaction of cross x postnatal diets (F3)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data represented as grams per 100 gram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

Table 3.15 Female F3 generation body composition 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Body wt (g)	Organ weight/body weight (%)									
					Liver	Left kidney	Right kidney	Heart	Lung	Brain	Thymus	Spleen	Gonadal fat pad	Perirenal fat pad
F3	Con x Con	Chow	9	255.9 ±9.71	4.77± 0.12	0.38± 0.01	0.40± 0.01	0.43± 0.02	0.71± 0.02	0.65± 0.05	0.17± 0.01	0.26± 0.01	0.69± 0.05	0.47± 0.07
	Con x Con	HF	9	236.1 ±7.46	4.89± 0.11	0.43± 0.01	0.46± 0.01	0.44± 0.01	0.89± 0.04	0.73± 0.04	0.17± 0.01	0.28± 0.02	0.96± 0.10	0.65± 0.10
	MLP x MLP	Chow	8	266.8 ±18.00	4.35± 0.10	0.38± 0.01	0.40± 0.01	0.41± 0.01	0.76± 0.03	0.64± 0.03	0.16± 0.01	0.25± 0.02	0.68± 0.08	0.56± 0.08
	MLP x MLP	HF	8	238.7 ±3.62	4.67± 0.17	0.40± 0.01	0.42± 0.01	0.42± 0.01	0.89± 0.04	0.69± 0.03	0.16± 0.01	0.28± 0.01	0.90± 0.08	0.71± 0.06
P for effect of maternal cross (F3)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F3)				P<0.05	P<0.05	P<0.05	P<0.05	NS	P<0.05	P<0.05	NS	NS	P<0.05	P<0.05
P for interaction of cross x postnatal diets (F3)				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data represented as grams per 100 gram body weight. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

Figure 3.6 Total abdominal fat deposition F3 generation

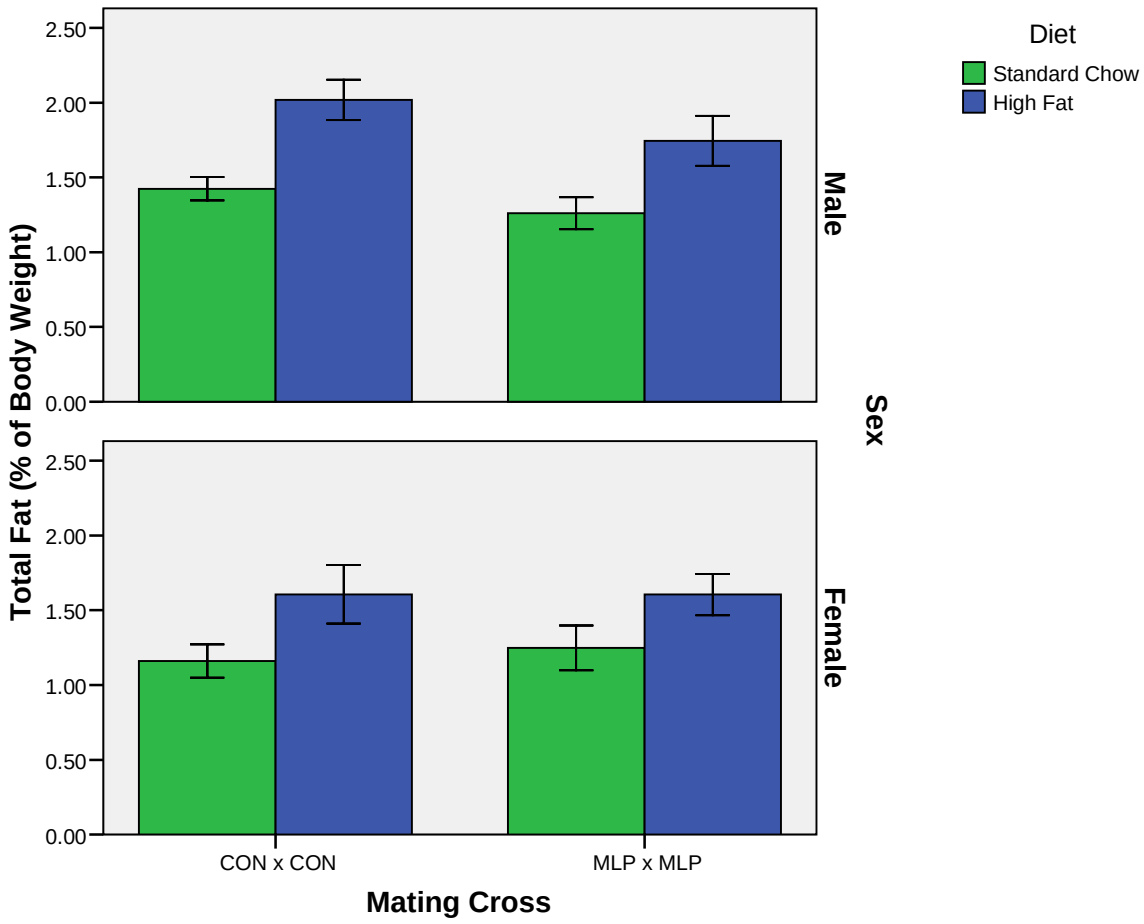


Figure 3.6 Total abdominal fat represents combined gonadal and perirenal fat deposits. Data is shown as mean ± SEM, for Con x Con male chow (n=11); male HF (n=9); female chow (n=9) and female HF (n=9), MLP x MLP male chow (n=6); male HF (n=8); female chow (n=8) and female HF (n=8). A significant effect of diet ($P<0.05$) was noted, Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to LP diet *in utero*.

3.8 Circulating metabolites

3.8.1 F1 generation

Table 3.16 shows circulating metabolite concentrations in the F1 generation at 10 weeks of age. Non-fasted plasma glucose concentrations were similar in animals exposed to both control and MLP diets *in utero*. Concentrations in female animals were comparable to concentrations in male animals. Similarly triglyceride, cholesterol and insulin concentrations were also unaffected by the maternal diet. Concentrations of these metabolites remained comparable in males and females regardless of the maternal diet *in utero.* Non-fasted insulin concentrations did not differ between sexes and values remained around the 3000 pmol/L level. Triglyceride and cholesterol concentrations in female offspring however were lower than in males ($P<0.05$) by approximately 20-40%.

With postnatal HF feeding, glucose and triglyceride concentrations remained similar to chow fed controls in both male and female animals. Cholesterol concentrations showed an almost two-fold increase ($P<0.05$) in male HF animals compared to chow controls, irrespective of the maternal diet. Female animals exhibited the same response to HF. However, the increase was much less pronounced than in males. The effect of postnatal HF feeding on plasma insulin concentrations was not measured. Throughout this study all circulating metabolite levels monitored were unaffected by the maternal diet. Triglyceride and cholesterol concentrations were higher in male animals compared to female animals. Postnatal HF feeding resulted in higher cholesterol levels. In F1 animals the prenatal diet had no effect on the monitored circulating metabolites.

Table 3.16 Circulating metabolites F1 generation 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Males				*n*	Females			
				Glucose mmol/L	Triglyceride mmol/L	Cholesterol mmol/L	Insulin pmol/L		Glucose mmol/L	Triglyceride mmol/L	Cholesterol mmol/L	Insulin pmol/L
F1	Control	Chow	11	9.198± 1.749	0.651± 0.152	1.747± 0.111	3514± 579	9	8.854± 1.459	0.514± 0.142	1.621± 0.071	2699± 345
	Control	HF	9	12.307± 1.646	0.566± 0.129	2.431± 0.232	-	10	9.645± 1.193	0.111± 0.103	1.511± 0.138	-
	MLP	Chow	8	10.296± 1.636	0.527± 0.078	1.589± 0.210	2474± 273	9	9.857± 1.697	0.303± 0.092	1.580± 0.190	2340± 326
	MLP	HF	9	8.929± 0.814	0.926± 0.198	2.647± 0.186	-	11	9.699± 0.894	0.288± 0.113	1.668± 0.089	-
P for effect of maternal diet (F1)				NS	NS	NS	NS	-	NS	NS	NS	NS
P for effect of postnatal diet (F1)				NS	NS	P<0.05	-	-	NS	NS	P<0.05	-
P for interaction of maternal x postnatal diets (F1)				NS	NS	NS	-	-	NS	NS	NS	-
P for effect of sex (F1)				NS	P<0.05	P<0.05	NS	-	NS	P<0.05	P<0.05	NS

Data shows mean ± SEM for *n* observations per group. NS= not significant.

3.8.2 F2 generation

Table 3.17 shows circulating metabolite concentrations in the F2 generation at 10 weeks of age. As in the F1 generation, non-fasted plasma glucose concentrations were similar in all animals regardless of the mating cross. Similarly cholesterol levels were also unaffected by mating cross. Concentrations of these two metabolites were similar in male and female animals. Like glucose and cholesterol concentrations, non-fasted insulin concentrations were similar in all mating crosses. However concentrations in male animals were significantly higher (45%, $P<0.05$) than in females. Triglyceride concentrations were significantly higher ($P<0.05$) in all MLP mating crosses compared to Con x Con in both male and female animals.

With feeding of the HF postnatal diet, glucose and cholesterol concentrations remained similar to those noted in chow fed animals (both sexes). Triglyceride levels however were lower ($P<0.05$, approximately 50%) in HF fed male animals compared to controls. The same trend was noted in the female animals ($P<0.05$) but the decrease was much less pronounced (approximately 7%). As in the F1 generation, the effect of postnatal HF feeding upon plasma insulin concentrations was not measured. Throughout this study circulating non-fasted glucose, non-fasted insulin and cholesterol concentrations were similar regardless of mating cross. Triglyceride concentrations were higher in all MLP mating crosses. Triglyceride concentrations were higher in males than in females and with postnatal HF feeding triglyceride levels decreased in both male and female animals. In F2 animals, only triglyceride concentrations were influenced by the fetal origins of the parents and therefore the original MLP dietary intervention.

Table 3.17 Circulating metabolites F2 generation 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Males				*n*	Females			
				Glucose mmol/L	Triglyceride mmol/L	Cholesterol mmol/L	Insulin pmol/L		Glucose mmol/L	Triglyceride mmol/L	Cholesterol mmol/L	Insulin pmol/L
F2	Con x Con	Chow	11	11.184± 1.664	0.889± 0.123	1.344± 0.154	3927± 492	11	12.032± 1.048	0.377± 0.077	1.646± 0.176	2813± 379
	Con x Con	HF	11	12.877± 1.740	0.703± 0.096	2.070± 0.232	-	11	11.217± 1.086	0.237± 0.069	1.787± 0.127	-
	Con x MLP	Chow	8	10.458± 1.785	1.168± 0.152	1.464± 0.068	4625± 818	8	12.411± 2.020	0.282± 0.094	1.555± 0.187	2559± 302
	Con x MLP	HF	8	8.174± 1.807	0.697± 0.151	1.983± 0.172	-	8	12.199± 0.807	0.309± 0.078	1.502± 0.103	-
	MLP x Con	Chow	6	13.184± 2.001	1.287± 0.272	1.362± 0.179	4750± 1084	10	10.514± 2.315	0.600± 0.123	1.602± 0.173	3118± 288
	MLP x Con	HF	7	9.256± 2.399	0.544± 0.074	2.052± 0.145	-	8	10.783± 2.583	0.276± 0.094	1.723± 0.216	-
	MLP x MLP	Chow	13	11.522± 1.439	1.003± 0.201	2.055± 0.407	6182± 1102	7	13.666± 2.293	0.403± 0.174	1.912± 0.066	3552± 814
	MLP x MLP	HF	10	10.115± 1.298	0.552± 0.484	1.499± 0.152	-	11	13.016± 1.447	0.374± 0.200	1.270± 0.404	-
P for effect of cross (F2)				NS	P<0.05	NS	NS	-	NS	P<0.05	NS	NS
P for effect of postnatal diet (F2)				NS	P<0.05	NS	-	-	NS	P<0.05	NS	-
P for interaction of cross x postnatal diets (F2)				NS	NS	NS	-	-	NS	NS	NS	-
P for effect of sex (F2)				NS	P<0.05	NS	P<0.05	-	NS	P<0.05	NS	P<0.05

Data shows mean ± SEM for *n* observations per group. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

3.8.3 F3 generation

Table 3.18 shows the circulating metabolite concentrations in the F3 generation at 10 weeks of age. All monitored circulating metabolites including glucose, insulin, triglyceride and cholesterol concentrations were unaffected by mating cross and sex. Postnatal HF feeding had no effect on glucose and triglyceride concentrations, but cholesterol concentrations were approximately 25% higher ($P<0.05$) in HF-fed male animals. The effect of postnatal HF feeding upon plasma insulin concentrations was not measured in the F3 generation. Similar to the F1 generation all rats, circulating metabolite concentrations monitored were unaffected by mating cross and no effect of sex was noted. Postnatal HF feeding resulted in higher cholesterol levels. In F3 animals, the prenatal diet had no effect on the monitored circulating metabolites.

Table 3.18 Circulating metabolites F3 generation 10 weeks

Generation	Maternal diet/cross	Postnatal diet	*n*	Males				*n*	Females			
				Glucose mmol/L	Triglyceride mmol/L	Cholesterol mmol/L	Insulin pmol/L		Glucose mmol/L	Triglyceride mmol/L	Cholesterol mmol/L	Insulin pmol/L
F3	Con x Con	Chow	11	11.847± 1.031	0.617± 0.138	1.533± 0.185	5679± 1029	9	10.525± 1.031	0.334± 0.103	1.605± 0.181	4082± 483
	Con x Con	HF	9	8.833± 1.599	0.917± 0.124	2.225± 0.191	-	9	10.031± 1.248	0.065± 0.046	1.570± 0.228	-
	MLP x MLP	Chow	6	9.028± 1.237	0.500± 0.138	1.650± 0.239	3875± 389	8	11.536± 4.035	0.485± 0.140	1.403± 0.102	3698± 436
	MLP x MLP	HF	8	7.780± 1.099	0.732± 0.143	2.022± 0.184	-	8	9.491± 1.245	0.181± 0.073	1.508± 0.129	-
P for effect of cross (F3)				NS	NS	NS	NS	-	NS	NS	NS	NS
P for effect of postnatal diet (F3)				NS	NS	P<0.05	-	-	NS	NS	P<0.05	-
P for interaction of cross x postnatal diets (F3)				NS	NS	NS	-	-	NS	NS	NS	-
P for effect of sex (F3)				NS	NS	NS	NS	-	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NS= not significant.

3.9 Discussion

3.9.1 Summary of Findings

Studies of the feeding of a low protein diet throughout gestation and the subsequent effects upon food intake, body composition and circulating metabolites in the F1 generation are well documented (Bellinger, 2005; Bellinger and Langley-Evans, 2005; Bellinger *et al.*, 2004; Erhuma *et al.*, 2007a; Erhuma *et al.*, 2007b; Vickers *et al.*, 2000). The data in this chapter shows for the first time the impact the original protein restriction has upon body composition and feeding behaviour in the F2 and F3 generations (Table 3.19).

Table 3.19 Summary of findings from animals culled at 10 weeks.

	Generation		
	F1	**F2**	**F3**
Body weight	Unchanged by maternal diet and postnatal HF feeding at 10 weeks.	Unchanged by mating cross and postnatal HF feeding at 10 weeks	Unchanged by mating cross, decreased with postnatal HF feeding at 10 weeks
Food intake	Unchanged by maternal diet, decreased with HF feeding.	Unchanged by mating cross, decreased with HF feeding.	Unchanged by mating cross, decreased with HF feeding.
Brain size	Unchanged by maternal diet and postnatal HF feeding at 10 weeks.	Unchanged by mating cross, increased with postnatal HF feeding at 10 weeks	Unchanged by mating cross, increased with postnatal HF feeding at 10 weeks
Abdominal fat	Unchanged by maternal diet, increased with postnatal HF feeding at 10 weeks	Decreased in all MLP mating crosses, increased with postnatal HF feeding at 10 weeks.	Unchanged by mating cross, increased with postnatal HF feeding at 10 weeks
Glucose/ insulin	Unchanged by maternal diet and postnatal HF feeding at 10 weeks.	Unchanged by mating cross and postnatal HF feeding at 10 weeks.	Unchanged by mating cross and postnatal HF feeding at 10 weeks.
Cholesterol/ TAG	Unchanged by maternal diet, cholesterol increased with postnatal HF feeding at 10 weeks.	Triglycerides increased in male MLP mating crosses, decreased with postnatal HF feeding at 10 weeks.	Unchanged by mating cross, cholesterol increased with postnatal HF feeding at 10 weeks.

Table 3.19 shows a brief summary of findings from animals from F1, F2 and F3 generations culled at 10 weeks of age.

Table 3.19 shows a brief summary of the findings from the animals culled at 10 weeks of age. The most striking finding from the analysis of body composition and food intake data was, the emergence of a lean abdominal fat phenotype within the F2 generation in all MLP mating crosses, which was not present within either the F1 or F3 generations.

3.9.1.1 Effect of prenatal protein restriction and mating cross.

Both food (standard laboratory chow) and energy (MJ per kilogram bodyweight) intakes were unaffected by MLP restriction during gestation at all time points monitored. No difference between male and female animals was also observed. These findings were unexpected as previous studies using exactly the same MLP diet within our laboratory indicated that MLP exposed offspring consumed significantly less than controls when fed chow, but have greater energy intake when placed on a self selection diet (Bellinger and Langley-Evans, 2005). In these earlier studies food intake was monitored at 12 weeks of age, a substantially later time point than monitored within this study. As such the animals within the study by Bellinger and Langley-Evans (2005) were more mature animals. This could explain the discrepancy with the current study.

Although previous work from our laboratory has shown MLP restricted F1 generation animals exhibit reduced food intake levels compared to control animals, work by Zambrano and colleagues (2006) noted no difference between MLP and control groups at 14 weeks of age (Zambrano *et al.*, 2006). Feed

efficiency was also reduced in the study by Bellinger and colleagues (2005), implying that the animals had an increased capacity for thermogenesis or other forms of energy expenditure (Bellinger, 2005). There was no effect of maternal protein restriction on feed efficiency in the F1 generation within the present study. Studies by Vickers and colleagues showed that offspring from severely undernourished mothers had significantly elevated food intake from an early postnatal age. This phenotype continued with advancing age and was also amplified by hypercaloric nutrition (Vickers *et al.*, 2000). It is therefore not entirely clear whether maternal protein restriction impacts on food intake within the F1 generation as the literature is currently split. However it is apparent from the data within this chapter that there was no effect of maternal protein restriction on food intake, energy intake or feed efficiency in the F1 generation within the current investigation.

This study has for the first time monitored feeding behaviour in 3 subsequent generations of animals, to analyse the potential for trangenerational programming. F2 generation feed efficiency was unaffected by mating cross at 5 and 7 weeks of age, but by 9 weeks efficiency in all MLP mating crosses was reduced compared to controls. This suggested that body weight gain may be compromised, but results discussed later within this section show that this is not the case.

Zambrano and colleagues (2005) have previously recorded food intake in F2 generation offspring following protein restriction. They noted no difference in MLP mating cross offspring, but did not report data on energy or feed efficiency (Zambrano *et al.*, 2005). Importantly, the study by Zambrano and colleages differs in several ways to the work within this study. Firstly and most

importantly, for breeding of the F2 generation their study utilised proven male studs from outside the experiment. The experiment could only therefore analyse transmission of phenotypes along the maternal line. Our study analysed both paternal and maternal effects. We noted that food intake was only affected in the offspring of mating crosses involving MLP exposed male offspring. Secondly the compositions of the MLP diets within the two studies differ. The present study includes methionine, whereas the Zambrano *et al.*, (2005) diet contains cysteine. Although both are sulphur containing amino acids, excess methionine is thought to perturb metabolism of homocysteine, ultimately contributing to differential methylation of DNA. This is a possible mechanism driving intergenerational programming. Thirdly, animals within this study were singly housed at 5, 7 and 9 weeks of age while food intake was monitored. Animals within the Zambrano study were housed in single sex caging at 14 weeks of age, the amount of food consumed was averaged between the numbers of animals in each cage. This method lacks accuracy.

F3 generation offspring exhibited no differences in energy intake between any of the mating crosses, at any of the time points monitored. Feed efficiency at 9 weeks was reduced in the MLP x MLP mating cross compared to controls. As previously stated, this is the first time in-depth analysis of feeding behaviour and body composition has been monitored in the F2 and F3 generations following maternal protein restriction. As such there is little evidence with which to compare the results within this chapter. Perhaps most striking is the fact that effects emerge in the F2 generation, for example reduced feed efficiency in all MLP mating crosses, that are not seen within the F1 generation. This has

possible implications for body composition, which is discussed later within this chapter.

Body composition of the F1 generation following maternal protein restriction has been well documented. Previous studies have suggested that adult body composition does not appear to differ between MLP exposed animals and control animals, although there is some evidence that MLP exposed male rats deposit more gonadal fat with ageing (Bellinger *et al.*, 2004). Roach *et al.*, have also reported abnormalities in bone morphology in MLP animals (Roach *et al.*, 1999). Similar to previous findings, body composition within this study did not differ with exposure to MLP diet *in utero*. All organs and fat pads were of similar size irrespective of maternal diet. It is important to note that the effect of aging upon body composition was not investigated in the F1 generation within the current study. There was no difference in circulating metabolites in the F1 generation confirming previous findings.

F2 generation body composition at 10 weeks of age was similar to F1 generation animals and all organs were of similar size relative to body weight. Unexpectedly F2 generation animals exhibited a lower abdominal fat deposition in all MLP groups. Although MLP groups by 9 weeks of age showed reduced feed efficiency leading us to expect a smaller body due to slower growth, F2 MLP groups were of normal bodyweight at time of cull. This suggests F2 generation MLP groups may be programmed to avoid fat gain whilst retaining lean mass (lean phenotype). This may be driven by mechanisms such as increased physical activity or thermogenesis. By 1 year of age (data not shown), the lean phenotype observed in the 10 week animals had disappeared, animals from all MLP groups had comparable body weights and abdominal fat deposits

to Con x Con group animals. Previous research in the F1 generation has indicated that although initially, up to 1 year, MLP offspring appear to be resistant to obesity, by 18 months there is an abrupt change in metabolic profile. Insulin resistance and hypertriacylglycerolaemia result in the development of an age-dependant metabolic syndrome-like phenotype (Erhuma *et al.*, 2007a). It is possible that this age-dependant process was operating in both the F1 and F2 generation within the current investigation. However the phenotype remained unnoticed as the effect of aging was not monitored within the F1 generation.

Mating cross had no effect on body composition in the F3 generation, all organs and fat pad sizes relative to body weight were similar in control and MLP groups, this was unexpected as in the previous generation effects due to MLP mating cross were noted. The F3 generation MLP group had lower feed efficiency at 9 weeks of age than control animals and were of similar body weight at time of cull. This suggests that the protective processes operating in the F2 were still operating, but less effectively. There was no change in body composition at 1 year of age.

3.9.1.2 Postnatal high fat feeding.

The primary aim of feeding a postnatal HF diet was to unmask any programmed hyperphagia in MLP crosses of the F2 and F3 generations. Previous studies have indicated that MLP exposed animals (F1) exhibit altered appetite preferences, with a preference for higher fat, more energy dense food types (Bellinger *et al.*, 2004). HF diets have been routinely used by several research groups in order to induce obesity following maternal dietary change (Ortmann *et*

al., 2003; Ozanne *et al.*, 2004; Vickers *et al.*, 2000). In contrast to previous research, the introduction of a HF diet at 4 weeks postnatal age to the F1 generation did not promote hyperphagia in MLP offspring, Food intake (grams per day per kilogram body weight) was substantially reduced at all time points monitored. Energy intakes initially reduced (5 weeks) with the introduction of the novel HF diet. However, by 7 weeks of age, energy intakes were similar to animals maintained on a standard laboratory chow postnatal diet irrespective of prenatal exposure to MLP. The initial reduction in energy intake was probably due to the palatability of the food - animals were only weaned onto the HF diet at 4 weeks of age, and therefore had possibly not fully become accustomed to the new diet. Texture of the diet is another possibility - the HF diet utilised with this study is very soft, possibly impacting upon energy intakes. It is also well known that young rodents very effectively regulate food intake to maintain energy balance (Woods and Strubbe, 1994). The observed lower intake probably reflects this response. The reason as to why the feeding of a HF diet did not promote hyperphagia in the MLP offspring after habituation to the HF diet is unknown. However, it is likely that the palatability of the diet is the key issue. Although Bellinger and colleagues (2005) used a HF diet with exactly the same composition, it must be noted that access to the HF diet in this study was part of a self- selection protocol whereby animals were offered free access to a HF diet, a high carbohydrate diet and a high protein diet (Bellinger., 2005). Despite the differences to previous research, the feed efficiency (weight gained per unit of energy intake) of animals maintained on a HF postnatal diet in the F1 generation roughly doubled. However, once again there was no interaction with maternal diet.

Unexpectedly, food intake of the F2 generation differed to that of the F1 generation, although food intakes similarly decreased in all groups when fed a HF diet. , An interaction between maternal diet/ mating cross and postnatal diet was noted at 5 and 7 weeks of age, which was absent in the F1 generation. Animals from the MLP x Con cross were hyperphagic compared to other crosses when maintained on a postnatal HF diet. Energy intakes however remained similar at all time points monitored and, although as in the F1 generation feed efficiency was higher in postnatal HF animals compared to chow, no interaction with mating cross was noted at 5 and 7 weeks of age. However at 9 weeks of age an interaction of maternal cross and postnatal diet was noted, with feed efficiency substantially decreasing in males from the MLP x Con cross compared to all the other F2 crosses.

F3 generation food intake was similar to the F1 generation, with no interaction of maternal diet/ cross noted at any time point and the introduction of a postnatal HF diet reduced food intakes in comparison to chow animals. Energy intakes were similar at 5 and 7 weeks of age, whereas previous generations had noted a consistent reduction in energy intakes at 5 weeks, suggesting that the introduction of a novel diet with a different palatability was not an issue for the F3 generation. However, at 9 weeks of age energy intakes in HF animals were reduced regardless of maternal cross. The reason why the reduction in energy intake was observed at a later time point in F3 animals is unknown, but this may imply possible changes to the central nervous system and the hypothalamus. The responses to the feeding of a HF diet postnatally differed across generations, but the reason for these differences remains unknown. As there was no interaction with maternal diet/ cross, these are essentially different batches of Wistar rats

and it would therefore be expected that all three generations would have similar responses to a postnatal HF challenge. There is, however, one concordant theme running through each generation and that is that the HF diet utilised within this study failed to induce hyperphagia.

As expected due to a higher feed efficiency, the subsequent effects of postnatal HF feeding in the F1 generation mainly impacted upon abdominal fat deposition. Gonadal and perirenal fat pad size was greater in animals on a HF postnatal diet at 10 weeks of age. However animals were of similar body weight at time of cull, suggesting that HF fed animals sacrificed lean tissue accretion for fat deposition during growth.

As noted in the F1 generation, postnatal HF feeding in the F2 mainly impacted upon abdominal fat deposition. Although body weight remained similar, fat pad size was greater in all mating cross groups. The lean phenotype observed in the MLP groups on postnatal chow diet remained even with HF feeding. This further suggests that the F2 MLP groups possessed an adaptive mechanism to cope with excessive energy in the diet. As suggested earlier this is possibly due to enhanced physical activity or thermogenesis.

As in the previous generations, HF feeding in the F3 generation resulted in greater abdominal fat deposition in combination with greater feed efficiency. Body weight however was significantly reduced and all organ sizes relative to body weight, except for the thymus, spleen and heart were greater than in chow fed animals, suggesting growth was somehow restricted in lieu of fat deposition.

3.9.1.3 Conclusion

The data within this chapter suggests that the HF diet failed to unmask any programmed hyperphagia as previously observed within the work of Bellinger and colleagues (2005). In contrast, animals demonstrated hypophagia when offered a HF diet, perhaps due to the poor palatability of the diet or as an attempt to regulate food intake to maintain energy balance.

The development of a lean phenotype within the F2 generation, which remains evident regardless of a postnatal HF challenge, suggests that the F2 MLP groups possessed an adaptive mechanism to cope with excessive energy in the diet via either enhanced physical activity or thermogenesis. However, the reason why responses varied across generations remains unknown. In order to follow up data from this chapter, an investigation of energy expenditure is essential as differences in energy intake and feed efficiency were absent and do not explain the emergence of the F2 lean phenotype.

4.0 Prenatal protein restriction, locomotor activity and behaviour

4.1 Introduction

The data described in chapter 3 suggested that F2 animals whose parents were exposed to protein restriction *in utero* exhibit reduced feed efficiency and appear to be resistant to abdominal fat deposition, even when consuming a HF diet. As no difference in energy intake was observed between these groups, we postulated that increased expenditure of energy through activity may be the underlying cause of this difference in phenotype.

Previous studies in our laboratory have shown that mature, low protein exposed animals actually deposit significantly more abdominal fat compared to control animals (Bellinger *et al.*, 2004). However, this is a phenotype that largely develops with senescence (between 12 and 18 months of age) (Erhuma *et al.*, 2007a). MLP-exposed animals have also been shown to have an increased preference for high fat, over high carbohydrate and high protein feed (Bellinger *et al.*, 2004). Previous experiments have examined the impact of timing of prenatal exposure to MLP upon energy expenditure. MLP exposure during the first week of gestation was found to be associated with the greatest reduction in energy expenditure when oxygen consumption was measured by indirect calorimetry (Bellinger, 2005). Providing a self-selection diet protocol at weaning to animals exposed to a MLP diet during gestation increased their fat preference. However if the introduction of the self-selection diet protocol is delayed until 6 months of age post-weaning, the effect is diminished (Bellinger *et al.*, 2004).

The effects of either a global or specific nutrient restriction during gestation and their subsequent effects on behaviour have been monitored by various authors. One of the first reports was by Jones and colleagues (1982) who studied the link

between restricted maternal nutrient supply and appetite and weight gain. They noted hyperphagia and subsequent weight gain in animals exposed to global nutrient restriction *in utero* and maintained on an energy dense diet postnatally (Jones and Friedman, 1982). Since this initial observation there has been a wealth of interest in how prenatal nutrition can impact upon later behaviour. One of the main areas of research has been upon the effect of calorie restriction both pre- and postnatally on indices of behaviour. Maternal calorie restriction during both gestation and /or lactation can have permanent effects on resulting offspring's responsiveness to novelty (Levitsky and Barnes, 1972), and social behaviour (Whatson and Smart, 1978). Caloric restriction has also been shown to enhance anxiety-like behaviours in rat offspring (Jaiswal *et al.,* 1996).

Despite growing interest, the mechanisms that programme behaviour associated with restricted maternal nutrition are poorly understood. Metabolic and physiological changes have been noted in animals exposed to calorie restriction both pre- and postnatally. Caloric restricted offspring have been shown to exhibit hypothalamic-pituitary-adrenal (HPA) hyperactivity under stress conditions (Sebaai *et al.,* 2004; Sebaai *et al.,* 2002). Most work on caloric restriction concentrates on a severe restriction of around 50% of *ad libitum* food intake, impacting on critical periods of brain development (Sebaai *et al.,* 2004). To date, data on moderate caloric restriction is unavailable. Differences in behaviour have also been noted in animals exposed to restriction of specific nutrients. Spasov and colleagues (2008) noted that rats fed a diet deficient in magnesium for 49 days developed anxiety-releated behaviours and depression (Spasov *et al.,* 2008). It is well-documented that stress during gestation may impact upon the offspring (Gotz *et al.,* 2008). Eseh and colleagues (2005) noted that a maternal diet deficient in iron resulted in offspring

with increased anxiety behaviours. This behaviour could be reversed with iron repletion, but some behaviour modifications such as altered exploratory behaviours, persisted (Eseh and Zimmerberg, 2005).

Several experimental situations are used in order to monitor animal behaviour and activity including the elevated plus maze, open field test, emergence test and the monitoring of locomotor activity. Previous studies with our laboratory have used assessments of locomotor activity to give a basic assessment of behaviour and physical activity (Bellinger *et al.*, 2006). Within the previous chapter it was noted that F2 animals whose parents were exposed to protein restriction *in utero* exhibit reduced feed efficiency and appear to be resistant to abdominal fat deposition, even when consuming a HF diet, possibly suggesting increased activity. The locomotor activity test was used in this chapter to analyse if there were any alterations in behaviour or locomotor activity between experimental groups that would explain this phenotype. The main hypothesis of this chapter is that F2 MLP mating cross animals have increased levels of activity (measured by analysis of locomotor activity) compared to their control counterparts, which ultimately contributes to the lean phenotype observed within the previous chapter.

4.2 Objectives

The work described within this chapter will examine the effects of a prenatal protein restriction upon locomotor activity in rats of the F1, F2 and F3 generations. The specific aim of the chapter was:

- To assess whether MLP groups within the F2 generation exhibit differing behaviour and levels of activity that might be related to the lean phenotype observed in the previous chapter.

4.3 Materials and Methods

4.3.1 Locomotor Activity

Locomotor activity (section 2.5.1) was monitored in the NC sub-group in the F1, F2 and F3 generations at both 4 and 10 weeks of age using a Linton AMIU53 Infrared Activity Monitor (Linton Instruments, Diss, UK). The first 30 minutes of a 90 minute test were used to assess the response to a novel environment, since animals were transferred from their home cage to the test cage. After 30 minutes they were regarded as habituated to the test conditions. Locomotor activity was only assessed during the 12-hour light phase.

4.3.2 Statistical analysis

All data is presented as mean ± SEM. All data was analysed using the statistical package for social sciences (SPSS) version 14. Differences between groups

were assessed using a mixed model ANOVA (fixed factors, maternal diet, sex, diet and age), $P<0.05$ was considered as significant.

4.4 Results

4.4.1 Week 4 Locomotor activity

4.4.1.1 F1 generation

Table 4.1 shows locomotor activity in the F1 generation animals at 4 weeks of age. Novel activity (breaking any beam on the lower level within 1 second), novel rearing (breaking a beam on the upper level recorded in 1 second intervals) and novel mobility (breaking more than one beam on the lower level, within 1 second). Counts were similar in animals exposed to control and MLP diets *in utero*. In both maternal dietary groups counts were similar in both male and female animals. When fed a HF diet novel activity, rearing and mobility counts all remained similar to values seen in the chow controls, irrespective of maternal dietary group.

Habituated activity, rearing and mobility counts are also shown within Table 4.1. No effect of maternal dietary group was noted. Counts in male and female animals were also comparable. When fed a HF diet, habituated activity counts were greater ($P<0.001$) approximately two-fold in the animals compared to postnatal chow animals, regardless of maternal diet *in utero* and sex. Habituated rearing counts remained similar in both postnatal dietary groups irrespective of maternal diet or sex. However, similar to activity levels, habituated mobility counts were greater ($P<0.001$) approximately two-fold when comparing rats fed a postnatal HF diet with thoase fed chow, irrespective of maternal diet and sex.

Table 4.1 Locomotor activity F1 generation 4 weeks of age.

				Locomotor Counts													
Generation	Maternal diet/cross	Postnatal diet	*n*	Males						*n*	Females						
				NA	NR	NM	HA	HR	HM		NA	NR	NM	HA	HR	HM
F1	Control	Chow	31	1037.8 ±43.4	510.8 ±59.3	383.0 ±23.7	281.0 ±49.2	183.1 ±83.8	32.2 ±9.8	29	1104.4 ±47.3	491.9 ±30.7	394.8 ±19.6	236.0 ±47.5	26.5 ±11.8	19.3 ±4.7
	Control	HF	10	949.8 ±76.4	472.6 ±125.4	335.8 ±37.4	431.1 ±105.6	214.1 ±138.3	69.9 ±16.3	10	1049.3 ±44.9	797.4 ±189.0	394.6 ±29.3	346.7 ±60.4	493.3 ±158.6	70.6 ±25.2
	MLP	Chow	28	1025.8 ±43.5	498.8 ±55.1	361.4 ±23.4	251.6 ±11.0	196.3 ±66.7	32.1 ±11.6	29	998.7 ±49.5	502.1 ±48.2	360.9 ±25.0	313.4 ±49.9	200.2 ±73.3	40.5 ±8.8
	MLP	HF	10	991.6 ±77.5	486.7 ±104.9	334.6 ±23.3	599.7 ±149.8	254.0 ±114.0	103.0 ±27.9	11	979.6 ±55.3	458.6 ±78.7	346.5 ±23.9	464.4 ±77.6	99.9 ±30.1	85.3 ±16.9
P for effect of maternal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F1)				NS	NS	NS	P<0.001	NS	P<0.001	-	NS	NS	NS	P<0.001	NS	P<0.001
P for interaction of maternal x postnatal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x maternal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x postnatal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as number of infra-red beams broken within 1 second. NA= novel activity, NR = novel rearing, NM = novel mobility, HA = habituated activity, HR = habituated rearing, HM = habituated mobility and NS= not significant.

4.4.1.2 F2 generation

Locomotor activity for the F2 generation at 4 weeks of age is displayed within Table 4.2. In the novel period, activity, rearing and mobility counts were unaffected by mating cross. Counts in male and female animals were also similar, regardless of mating cross. When fed a HF diet, novel activity and rearing levels remained similar to postnatal chow animal levels irrespective of mating cross. Novel mobility levels however, were ($P<0.05$) approximately 5% lower in all mating crosses regardless of sex, except the Con x Con male animals which had 15% greater mobility when fed HF relative to chow.

Habituated activity, rearing and mobility (Table 4.2) were also unaffected by mating cross. Counts were similar in male and female animals. When fed the postnatal HF diet, habituated activity and rearing levels remained similar to postnatal chow animals, however habituated mobility counts were ($P<0.05$) approximately 45% higher in comparison to postnatal chow fed animals. This was evident in both male and female animals irrespective of mating cross.

Table 4.2 Locomotor activity F2 generation 4 weeks of age.

				Locomotor Counts													
Generation	Maternal diet/cross	Postnatal diet	*n*	Males						*n*	Females						
				NA	NR	NM	HA	HR	HM		NA	NR	NM	HA	HR	HM	
F2	Con x Con	Chow	33	1001 ±77.11	347.5 ±79.3	318.7 ±48.1	407.9 ±115.2	80.8 ±22.9	50.4 ±13.1	33	1001.8 ±64.1	420.9 ±59.9	354.6 ±39.6	281.6 ±54.6	33.3 ±20.6	31.2 ±15.3	
	Con x Con	HF	11	1042.5 ±104.6	336.6 ±55.9	364.6 ±52.7	654.4 ±147.9	71.6 ±26.2	60 ±11.4	11	1085.1 ±39.6	391.8 ±40.8	338.6 ±29.9	477.2 ±114.9	88.8 ±52.4	47.1 ±13.7	
	Con x MLP	Chow	24	1039 ±85.6	520.8 ±95.4	362.3 ±38.2	479.4 ±150.3	209.9 ±136.5	33.8 ±11.4	24	1133.7 ±76.4	708.8 ±137.1	396.8 ±47.7	443.5 ±128.8	190 ±141.5	51 ±18.7	
	Con x MLP	HF	8	960.1 ±42.1	623 ±147.7	330.9 ±22.4	417.6 ±126.4	342 ±176.5	81.63 ±31.7	8	887.3 ±89.9	447.5 ±99.5	290.2 ±48	353.7 ±80.4	367.2 ±286.3	67.2 ±22.7	
	MLP x Con	Chow	23	1063.8 ±54.8	483.1 ±44.8	409.6 ±28.1	177.9 ±15.9	6.1 ±2.5	13.7 ±3.4	26	1054.4 ±71.8	528.9 ±105.7	379.1 ±26.6	313.8 ±114.8	179.1 ±126.9	46.1 ±21.4	
	MLP x Con	HF	7	900.3 ±61.5	319 ±50.5	299.3 ±33	351.9 ±105.4	129 ±68.8	74.3 ±31.2	8	1010.1 ±61.9	444.1 ±82.1	338 ±30.3	288 ±92	132.4 ±63.7	50.4 ±25.3	
	MLP x MLP	Chow	35	1002.5 ±39.5	465.9 ±72.4	351.7 ±24.3	286.5 ±45.5	92.1 ±43.6	37.3 ±10.6	29	1015.5 ±63.5	506.3 ±86.1	366 ±34	298.8 ±92.7	47.5 ±16.6	53.4 ±24.9	
	MLP x MLP	HF	10	1022 ±81.3	437.6 ±142.1	334.3 ±29.2	550.4 ±147.7	264.8 ±157.9	99.8 ±30.2	11	920.6 ±49.8	635.5 ±128.8	316.1 ±26.8	382.1 ±78.4	387.5 ±154.5	77.3 ±21.9	
P for effect of mating cross (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for effect of postnatal diet (F2)				NS	NS	P<0.05	NS	NS	P<0.05	-	NS	NS	P<0.05	NS	NS	P<0.05	
P for interaction of mating cross x postnatal diet (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for effect of sex (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for effect of sex x mating cross (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for effect of sex x postnatal diet (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	

Data shows mean ± SEM for *n* observations per group. . Data are represented as number of infra-red beams broken within 1 second. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NA= novel activity, NR = novel rearing, NM = novel mobility, HA = habituated activity, HR = habituated rearing, HM = habituated mobility and NS= not significant.

4.4.1.3 F3 generation

Table 4.3 depicts the locomotor activity of F3 generation animals at 4 weeks of age. As with the F1 and F2 rats, in the novel period activity, rearing and mobility counts were similar in both groups (Con x Con & MLP x MLP). Counts in male and female animals were also comparable. Postnatal HF feeding had no effects upon locomotor activity.

Once habituated the activity, rearing and mobility of the rats (Table 4.3) were also unaffected by mating cross, and sex. Postnatal HF feeding however did impact upon habituated rearing and mobility levels. Rearing behaviour was considerably more common ($P<0.05$) approximately 280% higher, and mobility was greater (approximately two-fold higher $P<0.05$) in animals fed the postnatal HF diet, compared to animals maintained on chow diet, irrespective of mating cross or sex. Habituated activity levels remained similar in all groups.

Table 4.3 Locomotor activity F3 generation 4 weeks of age.

				Locomotor Counts													
Generation	Maternal diet/cross	Postnatal diet	*n*	Males						*n*	Females						
				NA	NR	NM	HA	HR	HM		NA	NR	NM	HA	HR	HM
F3	Con x Con	Chow	31	1140.6 ±72.1	510.3 ±56.6	407.8 ±41.7	339.2 ±166.8	18.4 ±9.7	21.1 ±9.8	29	1021.9 ±37.7	456.6 ±61	394.7 ±24.1	293.7 ±54.7	45.1 ±25.5	29.6 ±13.5
	Con x Con	HF	11	1124.8 ±61.4	489.5 ±73.5	432.8 ±34.8	360.8 ±91.5	108.7 ±42.1	80.1 ±32.5	9	1040.8 ±67.3	491.9 ±61.4	399.1 ±49.7	439.4 ±115.5	278.3 ±92.1	104.9 ±42.4
	MLP x MLP	Chow	23	1031.1 ±51.7	511.6 ±43.8	387.1 ±45.6	500.4 ±149.1	54.5 ±27.0	101.6 ±58.5	24	1069.6 ±120.4	454.1 ±51.7	421.8 ±50.5	356.4 ±168.1	37.4 ±9.4	36.1 ±6.9
	MLP x MLP	HF	8	1131.9 ±100.8	387.3 ±48.7	381.6 ±45.6	498.3 ±119.9	136.6 ±60.1	103.4 ±30.2	8	953.3 ±59.4	373.8 ±39.6	343.4 ±29.9	374.5 ±87.9	144.5 ±51.8	88.5 ±27.3
P for effect of mating cross (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F3)				NS	NS	NS	NS	P<0.05	P<0.05	-	NS	NS	NS	NS	P<0.05	P<0.05
P for interaction of mating cross x postnatal diet (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x mating cross (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x postnatal diet (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. . Data are represented as number of infra-red beams broken within 1 second. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NA= novel activity, NR = novel rearing, NM = novel mobility, HA = habituated activity, HR = habituated rearing, HM = habituated mobility and NS= not significant.

4.4.2 10 week Locomotor Activity

4.4.2.1 F1 generation

Table 4.4 shows the locomotor activity of the F1 generation at 10 weeks of age. In the novel period, activity rearing and mobility counts were similar in animals exposed to control and MLP diets *in utero.* Novel activity ($P<0.001$) and mobility ($P<0.001$) values were higher by approximately 16% in females than in males. Postnatal HF feeding had no effect on novel activity, rearing or mobility counts, where values remained similar to chow fed animals. Once habituated, activity, rearing and mobility counts (Table 4.4) remained similar. Thus, as in 4 week old rats, at 10 weeks of age there was no effect of maternal diet *in utero* on locomotor activity in the F1 generation.

4.4.2.2 F2 generation

Locomotor activity of the F2 generation at 10 weeks of age is shown in Table 4.5. Novel activity, rearing and mobility counts were similar in animals from all mating crosses. Novel activity values were generally similar among male and female animals. However novel rearing levels were ($P<0.05$) approximately 20% lower in females compared to males on a chow postnatal diet, irrespective of mating cross. Novel mobility counts however were ($P<0.05$) approximately 25% higher in females compared to their male counterparts. Postnatal HF feeding had no effect on novel activity, rearing or mobility in the F2 generation, in either sex.

Table 4.5 also shows the habituated activity, rearing and mobility counts for the F2 generation animals. Similar to the novel period habituated activity rearing and mobility counts were unaffected by mating cross. There was also no difference

between male and female animals for habituated activity and rearing, but habituated mobility was greater ($P<0.001$) in females compared to male animals. There was no effect of postnatal HF feeding on habituated activity, but habituated rearing values were substantially higher (approximately three-fold, $P<0.001$) compared to levels observed in chow fed animals. Similarly, habituated mobility levels were also greater (2-3-fold, $P<0.001$) irrespective of sex and mating cross.

4.4.2.3 F3 generation

Table 4.6 shows locomotor activity of the F3 generation at 10 weeks of age. Novel activity and rearing were unaffected by mating cross. Novel mobility levels were ($P<0.05$) approximately 18% lower in the MLP x MLP mating cross, compared to the Con x Con controls. Novel activity, rearing and mobility levels were similar in male and females. Postnatal HF feeding also had no effect on novel activity, rearing or mobility levels.

In the habituated state, activity, rearing and mobility (Table 4.6) counts were all unaffected by mating cross. There was also no effect of sex. Postnatal HF feeding had no impact on habituated activity or mobility, but rearing was ($P<0.05$) approximately three-fold more prevalent in animals fed a postnatal HF diet compared to those fed a postnatal chow diet, irrespective of sex and mating cross.

Table 4.4 Locomotor activity F1 generation 10 weeks of age.

				Locomotor Counts													
Generation	Maternal diet/cross	Postnatal diet	*n*	Males						*n*	Females						
				NA	NR	NM	HA	HR	HM		NA	NR	NM	HA	HR	HM
F1	Control	Chow	31	1096.2 ±37.6	894.6 ±47.3	507.7 ±23.3	390.3 ±59.2	262.7 ±62.9	96 ±18.2	29	1280.3 ±35.7	798.4 ±36.7	586.9 ±22.4	472.9 ±69.9	285.1 ±60.6	122 ±25.5
	Control	HF	10	1035.1 ±52.8	740.4 ±159.4	398.6 ±41.6	600.8 ±146.8	516.1 ±157.2	151.7 ±34.4	10	1220 ±40.2	864.9 ±111.8	566.4 ±32.5	505.4 ±132.9	612.9 ±184.1	128.3 ±49.1
	MLP	Chow	28	1148.5 ±40.6	971.9 ±55.2	516.4 ±21.9	383.5 ±79.5	530.5 ±128.8	73.8 ±15.5	29	1263.6 ±35.1	865.2 ±51.2	594.3 ±23.5	525.8 ±71.5	389.8 ±84.3	149.6 ±25.6
	MLP	HF	10	1071.1 ±41.8	784.8 ±119.7	460.4 ±28.4	532.1 ±79.1	443.6 ±125.4	143.9 ±38.1	11	1347.9 ±42.5	813.5 ±108.1	626.6 ±27.3	548.3 ±128.7	427.1 ±143.3	128.5 ±43.1
P for effect of maternal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex (F1)				P<0.001	NS	P<0.001	NS	NS	NS	-	P<0.001	NS	P<0.001	NS	NS	NS
P for interaction of maternal x postnatal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x maternal diet (F1)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x postnatal diet (F1)				NS	NS	P<0.05	NS	NS	NS	-	NS	NS	P<0.05	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. Data are represented as number of infra-red beams broken within 1 second. NA= novel activity, NR = novel rearing, NM = novel mobility, HA = habituated activity, HR = habituated rearing, HM = habituated mobility and NS= not significant.

Table 4.5 Locomotor activity F2 generation 10 weeks of age.

				Locomotor Counts													
Generation	Maternal diet/cross	Postnatal diet	*n*	Males						*n*	Females						
				NA	NR	NM	HA	HR	HM		NA	NR	NM	HA	HR	HM
F2	Con x Con	Chow	33	1102.6 ±75.6	894 ±98.1	454.2 ±50.4	325 ±54.8	334.4 ±140.2	53.2 ±13.9	33	1199 ±41.3	789 ±70.1	529.5 ±22.3	436.5 ±197.4	186.9 ±96.4	42.9 ±27
	Con x Con	HF	11	1051.3 ±37.2	945.2 ±138.3	387.4 ±37.4	432.5 ±102.2	602.8 ±230	48.2 ±15.2	11	1188.8 ±75.6	846.9 ±73.9	534.3 ±38.6	348.6 ±81.6	319.1 ±120.2	91.4 ±25.5
	Con x MLP	Chow	24	994.1 ±53.5	816.6 ±83.1	379.5 ±26.8	220.4 ±48.3	24.1 ±6.9	13.4 ±4.4	24	1226.4 ±33.5	678 ±52.7	568.6 ±25.8	401.7 ±99.2	141.4 ±59	99.4 ±39.3
	Con x MLP	HF	8	1065 ±128.9	666.5 ±116.3	380.6 ±52.5	581.5 ±219.4	293.8 ±167.6	99.5 ±51.7	8	1012.6 ±71.7	611.4 ±66.6	423.9 ±33.5	369.3 ±139.9	197.1 ±70.4	59.6 ±19.6
	MLP x Con	Chow	23	896.4 ±86.3	718 ±97.8	381.8 ±45.1	113.2 ±56.8	194.4 ±176.1	20.8 ±13.9	26	996.6 ±74	580.9 ±69.8	426.9 ±38.1	162.4 ±33	117.8 ±65.9	23.8 ±8.3
	MLP x Con	HF	7	1002.7 ±41.6	860.2 ±67.6	424.5 ±26.5	264.7 ±71.2	609.8 ±218.9	57 ±22.7	8	1101.1 ±104.6	819.4 ±128.1	467.7 ±55.5	349.7 ±138.7	368.1 ±151.9	61.3 ±27.4
	MLP x MLP	Chow	35	1040.4 ±48.2	774.2 ±90.3	413.7 ±25.7	417 ±133.7	152 ±121.8	31.6 ±6.9	29	1138.6 ±97.4	613.7 ±74.9	520.7 ±50.2	353.3 ±109.1	302 ±224.8	83.4 ±40.1
	MLP x MLP	HF	10	1044.7 ±55.8	1010 ±132.5	393.7 ±26.9	392.8 ±100.9	660.4 ±202.9	51 ±23.9	11	1132.8 ±66.6	796.9 ±126.2	463.1 ±39.1	496.4 ±122.4	556.8 ±147.4	146.9 ±53.7
P for effect of mating cross (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of postnatal diet (F2)				NS	NS	NS	NS	P<0.001	P<0.001	-	NS	NS	NS	NS	P<0.001	P<0.001
P for effect of sex (F2)				NS	P<0.05	P<0.05	NS	NS	P<0.001	-	NS	P<0.05	P<0.05	NS	NS	P<0.001
P for interaction of mating cross x postnatal diet (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x mating cross (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
P for effect of sex x postnatal diet (F2)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS

Data shows mean ± SEM for *n* observations per group. . Data are represented as number of infra-red beams broken within 1 second. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NA= novel activity, NR = novel rearing, NM = novel mobility, HA = habituated activity, HR = habituated rearing, HM = habituated mobility and NS= not significant.

Table 4.6 Locomotor activity F3 generation 10 weeks of age.

				Locomotor Counts													
Generation	Maternal diet/cross	Postnatal diet	*n*	Males						*n*	Females						
				NA	NR	NM	HA	HR	HM		NA	NR	NM	HA	HR	HM	
F3	Con x Con	Chow	31	1132.4 ±46.7	738.9 ±53.5	489.8 ±26.6	261.8 ±52.9	101.3 ±40.5	51.2 ±20.5	29	1121 ±60.5	738.2 ±57.6	558.2 ±38.7	367.3 ±105.6	134.1 ±58.6	78.2 ±32.3	
	Con x Con	HF	11	1155.9 ±24.2	861.7 ±118.9	502.3 ±21.2	648.6 ±146.1	385.7 ±150.1	119.7 ±27.8	9	1225.7 ±45.8	1085.8 ±161.5	565.1 ±52.9	347.4 ±96.8	775 ±249.4	107.7 ±40.5	
	MLP x MLP	Chow	23	1075.3 ±103.1	441.8 ±82	398.8 ±60.5	149.5 ±35.4	15.3 ±12.9	9.7 ±3.3	24	953.4 ±56.6	496 ±78.5	395.9 ±46.3	181.9 ±53.3	52.5 ±30.8	34.3 ±15.8	
	MLP x MLP	HF	8	1053 ±90.5	762.5 ±110.9	437.8 ±61.5	378 ±134.3	414.6 ±142.9	104.3 ±50.5	8	1039.6 ±37.5	523.5 ±78.6	376.6 ±23.3	216.4 ±74.1	217.1 ±108.2	42.5 ±21.3	
P for effect of mating cross (F3)				NS	NS	P<0.05	NS	NS	NS	-	NS	NS	P<0.05	NS	NS	NS	
P for effect of postnatal diet (F3)				NS	NS	NS	NS	P<0.05	NS	-	NS	NS	NS	NS	P<0.05	NS	
P for effect of sex (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for interaction of mating cross x postnatal diet (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for effect of sex x mating cross (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
P for effect of sex x postnatal diet (F3)				NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	

Data shows mean ± SEM for *n* observations per group. . Data are represented as number of infra-red beams broken within 1 second. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*. NA= novel activity, NR = novel rearing, NM = novel mobility, HA = habituated activity, HR = habituated rearing, HM = habituated mobility and NS= not significant.

4.5 Discussion

4.5.1 Summary of findings

Investigations of the effects of caloric restriction and the feeding of a MLP diet *in utero* upon locomotor activity in the F1 generation have been previously documented (Bellinger *et al.*, 2006; Vickers *et al.*, 2003). The data presented in this chapter uniquely considers whether this original dietary insult has an effect on locomotor activity and behaviour in subsequent generations.

4.5.1.1 Summary of findings 4 week locomotor activity.

Table 4.7 Summary of findings at 4 weeks of age.

	Generation		
	F1	**F2**	**F3**
Novel activity	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet
Novel rearing	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet
Novel mobility	No change with maternal diet or with postnatal diet	No change with maternal diet, decreased with HF postnatal feeding	No change with maternal diet or with postnatal diet
Habituated activity	No change with maternal diet, increased with HF postnatal feeding	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet
Habituated rearing	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	No change with maternal diet, increased with HF postnatal feeding
Habituated mobility	No change with maternal diet, increased with HF postnatal feeding	No change with maternal diet, increased with HF postnatal feeding	No change with maternal diet, increased with HF postnatal feeding

Table 4.7 shows a brief summary of findings from the assessment of locomotor activity at 4 weeks of age in the F1, F2 and F3 generations.

Table 4.7 shows a summary of findings from the 4 week locomotor analysis. There was no effect of maternal diet/ mating cross on locomotor activity in either novel or habituated periods at 4 weeks of age, in any generation. Postnatal high fat feeding resulted in greater habituated activity and mobility in the F1 generation. However in the F2 generation HF feeding decreased novel and habituated mobility. In the F3 generation postnatal HF feeding resulted in higher habituated rearing and mobility counts. This data suggest that rats alter locomotor activity when challenged with a HF diet, but despite variation in the nature of this response between generations, there was no evidence of programming by protein restriction.

4.5.1.2 Summary of findings 10 weeks locomotor activity.

Table 4.8 Summary of findings at 10 weeks of age.

	Generation		
	F1	**F2**	**F3**
Novel activity	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet
Novel rearing	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet
Novel mobility	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	Decreased in MLP x MLP mating cross, no effect of postnatal diet
Habituated activity	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet	No change with maternal diet or with postnatal diet
Habituated rearing	No change with maternal diet or with postnatal diet	No change with maternal diet, increased with HF postnatal feeding	No change with maternal diet, increased with HF postnatal feeding
Habituated mobility	No change with maternal diet or with postnatal diet	No change with maternal diet, increased with HF postnatal feeding	No change with maternal diet or with postnatal diet

Table 4.8 shows a brief summary of findings from the assessment of locomotor activity at 4 weeks of age in the F1, F2 and F3 generations.

Table 4.8 shows a summary of the findings from the locomotor analysis of 10 week old animals. There was no effect of maternal diet/ mating cross on locomotor activity in either novel or habituated states, at 10 weeks of age, in the F1 or F2 generation. In the F3 generation novel mobility was decreased in the MLP x MLP group. Postnatal HF feeding had no impact upon the behaviour of the F1 generation at 10 weeks of age. The F2 generation exhibited higher habituated rearing and mobility. Postnatal HF diet resulted in greater habituated rearing counts in the F3 generation. These findings largely confirm those in younger animals, as there were few programmed changes in behaviour. Interestingly in the older rats the response to HF feeding seen in weanlings was largely absent.

4.5.2 Effect of prenatal protein restriction and mating cross on locomotor activity.

Over the last few decades there has been considerable interest in environmental stressors and the development of behavioural disorders. The most striking of these was the association between schizophrenia (Susser, 1996; Susser *et al.*, 1996) and affective disorder (Brown *et al.*, 2000) following early life exposure to the Dutch Hunger Winter. Maternal stress and lower socio-economic status in gestation have also been associated with attention deficit disorder and developmental delays in childhood (Biederman *et al.*, 1995; Milberger *et al.*, 1997; Stott, 1973). Indeed many variables have the potential to impact upon fetal development including, stress, malnutrition, disease susceptibility, drug use and

delivery complications, (Faraone and Biederman, 1998; Milberger *et al.*, 1997; Sprich-Buckminster *et al.*, 1993). The current study utilised analysis locomotor activity to investigate behaviour and activity within the F1, F2 and F3 generations.

The analysis of locomotor activity is used as an indication of spontaneous behaviour over a 90 minute period that is split into three stages (each 30 minutes), firstly, the novel period (first 30 minutes). Locomotor activity during this time is analysed in order to assess behaviour in a novel environment. Secondly, the habituated period, which is an average of the counts observed in the second and third stages. Locomotor activity during this time is used to assess habituated locomotor activity, for example how the animals behaves in the home cage.

Previous work within our laboratory has indicated that, at 13 months of age, female animals were more active than males in novel periods, and that rearing behaviours were significantly influenced by maternal diet. MLP exposed animals exhibited lower levels of rearing than controls (Bellinger *et al.*, 2006). Similarly Vickers and colleagues noted decreased activity in animals exposed to maternal undernutrition (Vickers *et al.*, 2003). As previously stated, rearing behaviour is often referred to as emotional behaviour, and is associated with stressful situations, such as being in a novel environment (Borta and Schwarting, 2005). This therefore suggests that animals exposed to protein restriction *in utero* are less likely to become stressed in novel situations, or that they have a reduced exploratory behaviour. Opinions about the effects of prenatal malnutrition are clearly split as Almeida and co-workers indicate that prenatal malnutrition enhances exploratory behaviour (Almeida *et al.*, 1996). Interestingly, recent

studies examining the impact of maternal cafeteria feeding to induce obesity during the pre-mating, pregnancy or suckling periods, has suggested offspring exposed to highly palatable diet during suckling show less anxiety when on a plus-maze or in an open field (Wright *et al.*, unpublished data).

Unlike previous studies, data from the current investigation indicated no effect of maternal diet or mating cross on F1 or F2 generation locomotor activity levels at either 4 or 10 weeks of age in either novel or habituated periods. Similar to the F2 generation, the F3 generation exhibited no maternal diet-related difference in locomotor activity in novel or habituated periods at 4 weeks of age, but by 10 weeks of age animals from the MLP x MLP mating cross were less mobile in the novel period than the Con x Con group. Although the results were unexpected, it is important to note that the present study differs in several ways to studies that have previously reported reductions in activity and rearing levels (Bellinger *et al.*, 2006; Vickers *et al.*, 2003). Firstly in the study by Vickers *et al.*, (2003) a global restriction of 30% was applied in pregnancy, compared to the 9% protein restriction used within the present study. Also timing and type of locomotor assessment was different, locomotor activity was measured at 5, 20 and 60 weeks of age using less sophisticated methodology than in the present study where assessments were made at 4 and 10 weeks of age. Similarly in the work by Bellinger and colleagues (2006), only animals subjected to a (9% casein) MLP diet during the final week of gestation exhibited decreased rearing counts. Animals were also assessed at 13 months of age so there is the possibility that MLP animal groups could develop differences in behaviour with aging. Another possible alternative is that differences in behaviour manifest at night (during darkness). During this investigation locomotor activity was only

monitored during daylight and this could possibly account for the differences between the studies.

One of the aims of the work presented in this chapter, was to assess whether differences in body composition noted in chapter 3 could be related to differences in locomotor activity and hence energy expenditure through physical activity. We had noted that F2 animals derived from parents originally exposed to a MLP diet *in utero*, exhibited a leaner body composition and a relative resistance to fat gain when fed a HF diet. This suggests that mechanisms are in place to expend energy without fat accretion, since food and energy intakes showed no evidence of programmed differences. The analysis of locomotor activity showed no difference in components of behaviour that might contribute to energy expenditure (activity & mobility). This indicates that other mechanisms, such as increased thermogenesis or BMR maintain the leaner body composition seen in these animals.

To date, there is very little literature about BMR in relation to fetal programming. Daenzer and coworkers (2002) considered the effect of increased maternal protein *in utero* on resting metabolic rate (RMR) in F1 generation animals and found that maternal protein exposure had no effect. However this study did report a decrease in both total and weight specific total energy expenditure (TEE), suggesting the cause may be due to thermogenesis or physical activity (Daenzer *et al.*, 2002). Similarly, studies within our laboratory have found no difference in RMR in MLP exposed animals (Bellinger, 2005; Ware, 2008). Unfortunately due to the complex study design and time limits we were unable to monitor RMR within the present study. Our data in the context of other work suggests that differences in body composition seen within the F2

generation must be due to some other mechanism such as non-exercise activity thermogenesis (Levine *et al*., 2006).

Uncoupling protein 1 (UCP1) expression is associated with thermogenic activity. Expression of UCP1 is most abundant in brown adipose tissue (BAT) (Power, 1989). It has been proposed that overall BAT mass may be related to UCP1 availability. Recent observations suggest that UCP1 expression in brown adipocytes within inguinal fat deposits are very sensitive to the nutritional environment, as global undernutrition during gestation severely suppresses UCP1 expression in the fat depots of mice (Kozak and Anunciado-Koza, 2008). Previous work within our laboratory has indicated that animals exposed to MLP have altered ratios of brown to white fat in the intrascapular depot and have significantly increased UCP1 expression in BAT (Bellinger, 2005). Scarpace and colleagues (1997) noted that increased UCP1 expression is associated with increased energy expenditure (Scarpace *et al.*, 1997). Recent studies in mice however, cast doubt on UCP1 as a mechanism protecting against fat gain, as UCP1 deficient mice have been shown to be resistant to diet induced obesity (Liu *et al.*, 2003). Although current opinion is split, these results suggest that within the present study, the "lean phenotype" observed within the F2 generation could be due to increased UCP1 expression. This mechanism enables animals to process excess energy less efficiently, reducing fat deposition.

4.5.3 Effect of postnatal HF feeding on locomotor activity and behaviour.

In the present study the postnatal feeding of a HF diet to rats of the F1 generation had no effect on activity, rearing or mobility in the novel period at

either 4 or 10 weeks of age. However, in the habituated state, activity and mobility levels were elevated in 4 week old animals. Previous data suggests that postnatal hypercaloric nutrition exacerbates sedentary behaviour (Vickers *et al.*, 2003), this was however data from prenatal globally restricted (30%) animals. In a recent study, mice selectively bred for a high level of wheel running were maintained on a HF diet (60%). Male animals were observed to reduce their food intake but still increased body fat, whereas females increased their ingested calories without any effects on body mass. Females increased daily energy expenditure, spontaneous activity (700%) and had a higher BMR (Vaanholt *et al.*, 2008). This data suggests that on feeding HF to rodents, both BMR and locomotor activity are altered in a sex specific manner in order to facilitate fat utilization.

To my knowledge no data currently exist with the wider body of literature regarding locomotor activity in programmed animals in the F2 and F3 generations. Our results show that at 4 weeks of age F2 animals on a HF postnatal diet exhibited increased mobility levels in both novel and habituated periods. However by 10 weeks of age both habituated rearing and mobility were increased. Similarly habituated rearing (4 & 10 weeks) and mobility (4 weeks) levels were higher in the F3 generation. This suggests that the activity was increased in animals on postnatal HF diet in order to shed excess energy imposed by the postnatal diet.

4.5.4 Conclusions.

Data from the previous chapter suggested an intergenerational effect of a MLP diet during gestation, impacting upon fat deposition in the F2 generation in

the absence of changes to energy intake between groups. Although the mechanism driving the observed lean phenotype remains unknown, we can exclude differences in activity, and so it is likely to involve thermogenesis and possibly UCP1 expression. Work presented in this thesis will now focus on the well-documented raised systolic blood pressure associated with the MLP diet.

5.0 Prenatal protein restriction and blood pressure.

5.1 Introduction

Findings from epidemiological associations between weight at birth and CVD (Barker *et al.*, 2002b; Barker *et al.*, 1993; Langley-Evans, 2006) are supported by animal experiments showing that nutrition *in utero* can programme adult blood pressure (Bergel and Belizan, 2002; Crowe *et al.*, 1995; Gambling *et al.*, 2003; Langley and Jackson, 1994). The feeding of MLP in rat pregnancy as reported within this investigation, has previously been shown to programme a lifelong elevation of systolic blood pressure (Langley-Evans *et al.*, 1996b; Langley-Evans *et al.*, 1996c; Langley-Evans *et al.*, 1994; Langley and Jackson, 1994). It is well documented that animals exposed to MLP diet *in utero* consistently exhibit raised systolic blood pressure by 4 weeks of age (Langley-Evans, 2001). The magnitude of the increase varies between 7 and 30 mmHg (Langley-Evans *et al.*, 1994; Langley and Jackson, 1994) and appears to be permanent, remaining elevated well into adulthood, in both male and female animals (Langley-Evans and Jackson, 1995; Nwagwu *et al.*, 2000). High blood pressure associated with the MLP diet is also found to coincide with a reduced heart rate (Nwagwu *et al.*, 2000). Previous studies within our laboratory have suggested that the timing of the MLP insult during gestation is not critical in the programming of blood pressure. Feeding MLP for single weeks of gestation, for example days 0-7, increased blood pressure to a similar level as that noted in animals restricted between days 8-14 (Langley-Evans *et al.*, 1996d). The final week of gestation (days 15-22), has been shown to be associated with a greater elevation of blood pressure. However, the greatest increase is seen when feeding a MLP diet throughout the whole of gestation (Langley-Evans, 2004). The effects of a MLP diet during gestation on blood pressure are not limited to rats. Studies in mice have

demonstrated that MLP diet throughout pregnancy also induces hypertension in the offspring (Dunn *et al.*, 2001). Current thinking is that protein restriction elicits an effect on blood pressure, at least in part, by altering the structure and vasculature of the kidney. Indeed, work within our laboratory has shown that rats exposed to a MLP diet *in utero* have a reduced nephron complement at the time of birth (Langley-Evans *et al.*, 2003; Langley-Evans *et al.*, 1999a). Martyn and Greenwald (2001) also hypothesised that programming of the vascular structure contributes to high blood pressure (Martyn and Greenwald, 2001). MLP feeding in gestation has been shown to induce noticeable changes within the ascending aorta such as altered elastin and collagen deposition from 4 weeks of age (Langley-Evans, 2004). The analysis of blood pressure was used in this investigation to asses if the hypertensive phenotype programmed by protein restriction was apparent in the F2 and F3 generations.

5.2 Objectives

The work described within this chapter will examine the effects of prenatal protein restriction upon systolic blood pressure in rats of the F1, F2 and F3 generations. The specific aims of the chapter are:

- To confirm previous findings on the effects of exposure to a MLP diet *in utero*, on the blood pressure and nephron complement of the F1 generation.
- To assess whether elevated blood pressure and perturbations in nephron complement in the F1 generation are transmitted to subsequent generations (F2 and F3).

5.3 Materials and Methods

5.3.1 Determination of Blood Pressure

As described in (section 2.5.2), blood pressure was determined in all animals using an indirect tail cuff method (Sherman and Langley-Evans, 1998). All rats were housed at 27°C, in the room where the measurements were made for at least 2 hours prior to testing. Blood pressure was determined at 4, 6 and 8 weeks of age. Measurements were taken in triplicate and a mean value was derived for each animal.

5.3.2 Nephron Number Determination

Nephron number (section 2.5.3), was determined using an adaptation of the acid maceration method (Welham *et al.*, 2002).

5.3.3 Statistical analysis

All data is presented as mean ± SEM. All data was analysed using the Statistical Package for Social Sciences (SPSS) version 14. Differences between groups were assessed using a mixed model ANOVA (fixed factors, maternal diet, sex, diet and age), $P<0.05$ was considered as significant.

5.4 Results

5.4.1 Blood pressure

5.4.1.1 F1 generation

Systolic blood pressure at 4 weeks of age in F1 generation offspring (Figure 5.1) was approximately 9% lower ($P<0.05$) in MLP exposed animals than in controls. Blood pressure was also similar in both male and female animals. However, by 6 weeks of age (Figure 5.1) this significant difference in blood pressure had disappeared and systolic blood pressure was similar in both maternal dietary groups. Male and female animals also remained comparable. At 8 weeks of age (Figure 5.1) blood pressure in the MLP group was significantly ($P<0.05$) higher (approximately 9mmHg) than in the control group (Con).

At 4 weeks of age (Figure 5.1) postnatal HF feeding significantly ($P<0.05$) lowered systolic blood pressure by approximately 10mmHg compared to chow fed animals. This occurred independent of sex. By 6 weeks of age (Figure 5.1) the effect of the HF diet was diminished and pressures in Chow and HF animals were similar. By 8 weeks of age postnatal HF feeding resulted in significantly higher ($P<0.05$) systolic blood pressure in MLP exposed animals regardless of sex (Figure 5.1).

Figure 5.1 F1 generation systolic blood pressure at 4, 6 and 8 weeks of age.

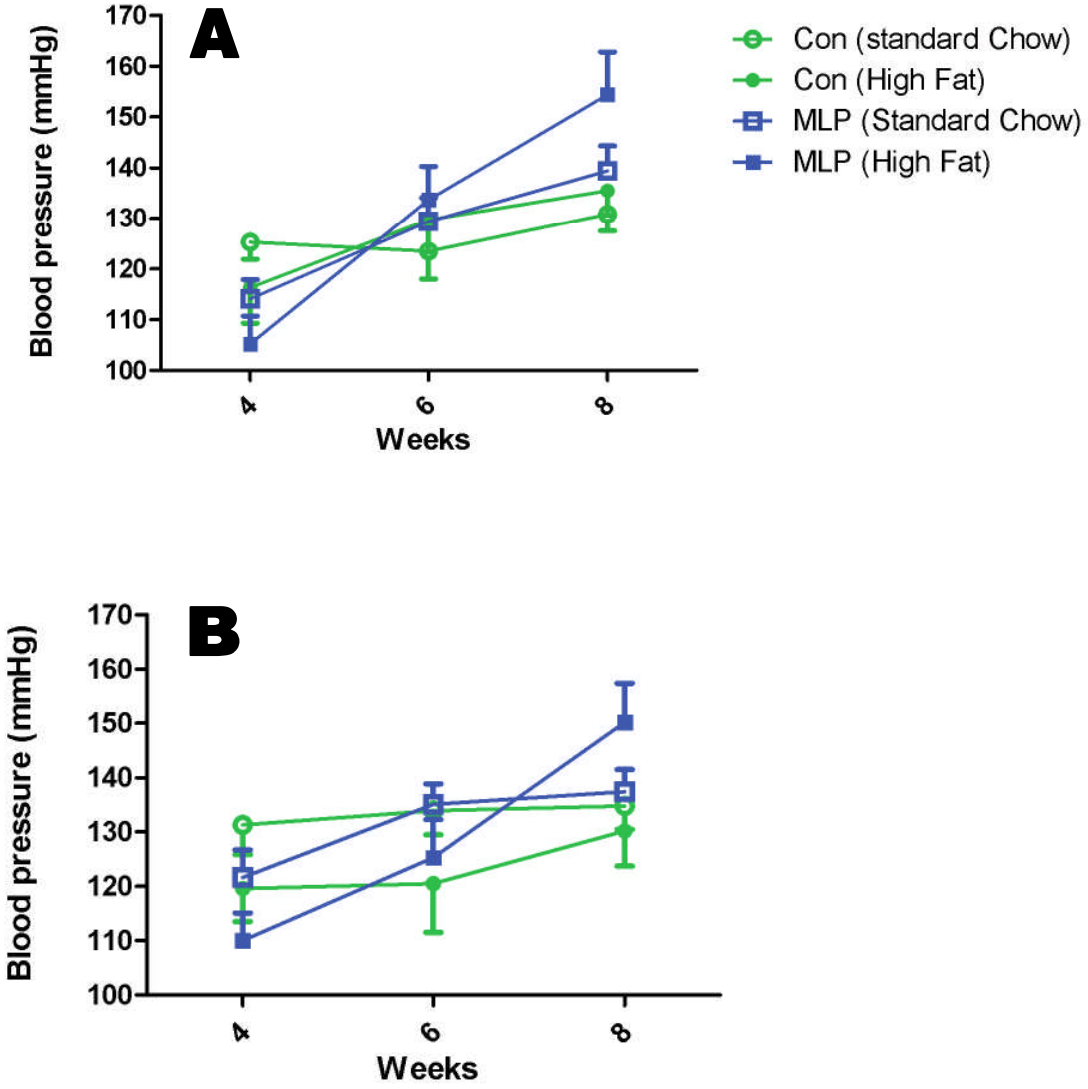


Figure 5.1 Data is shown as mean ± SEM. Graph A represents male systolic blood pressure at 4, 6 & 8 weeks of age. For Control chow (n = 31); Control HF (n = 10); MLP chow (n = 28); MLP HF (n = 10). Graph B represents female systolic blood pressure at 4, 6 & 8 weeks of age. For Control chow (n = 29); Control HF (n = 10); MLP chow (n = 29); MLP HF (n = 11). Significant effects of maternal diet ($P<0.05$) were noted at 4 and 8 weeks of age. Significant effects of postnatal diet ($P<0.05$) were noted at 4 weeks of age. Data analysed using a mixed model ANOVA.

5.4.1.2 F2 generation

Figure 5.2 shows the systolic blood pressure at 4 weeks of age in the F2 generation. Blood pressure was similar in all mating cross groups (approximately 130 mmHg). Blood pressure in male animals was comparable to that observed in female animals. At 6 weeks of age, (Figure 5.2) there was a significant influence of mating cross. All MLP mating crosses were approximately 4 mmHg higher ($P<0.05$) than the Con x Con mating cross. Pressures in male and female animals were similar. At 8 weeks of age (Figure 5.2) the trend of elevated blood pressure in the MLP mating crosses continued ($P<0.001$), MLP animals were approximately 5-10 mmHg higher than the Control mating cross (Con x Con). The feeding of a postnatal HF diet had no effect upon systolic blood pressure in the F2 generation at any of the time points in which systolic blood pressure was monitored.

Figure 5.2 F2 generation systolic blood pressures at 4, 6 and 8 weeks of age.

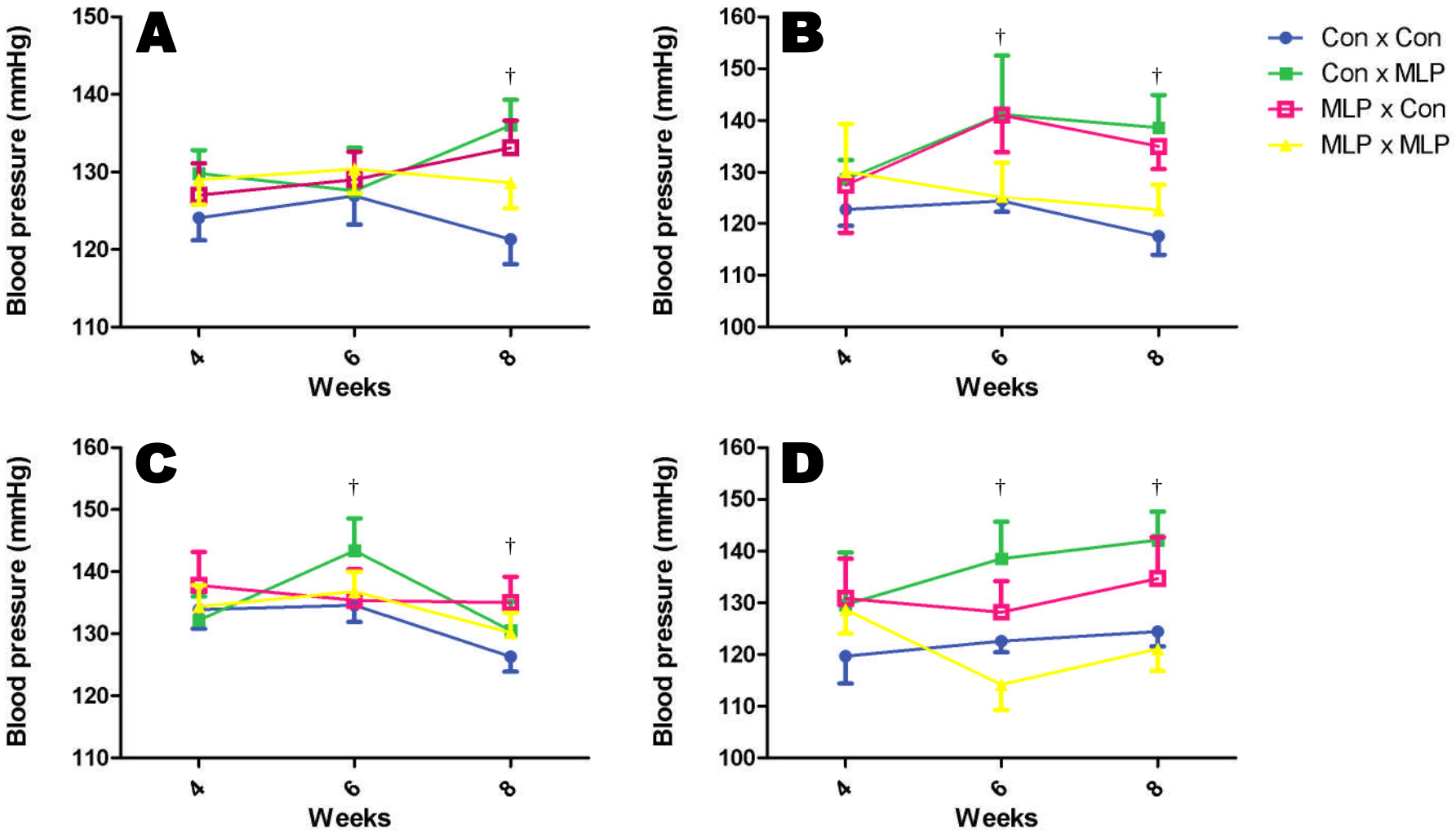


Figure 5.2 Data is shown as mean ± SEM. Graph A represents the systolic blood pressure at 4, 6 & 8 weeks of age of male animals maintained on a chow postnatal diet. For Con x Con (n = 33); Con x MLP (n = 24); MLP x Con (n = 23); MLP x MLP (n = 35). Graph B represents the systolic blood pressure at 4, 6 & 8 weeks of age of male animals maintained on a HF postnatal diet. For Con x Con (n = 11); Con x MLP (n = 8); MLP x Con (n = 7); MLP x MLP (n = 10). Graph C represents the systolic blood pressure at 4, 6 & 8 weeks of age of female animals maintained on a chow postnatal diet. For Con x Con (n = 33); Con x MLP (n = 24); MLP x Con (n = 26); MLP x MLP (n = 29). Graph D represents the systolic blood pressure at 4, 6 & 8 weeks of age of female animals maintained on a HF postnatal diet. For Con x Con (n = 11); Con x MLP (n = 8); MLP x Con (n = 8); MLP x MLP (n = 11). † indicates a significant effect of mating cross. Significant effects of mating cross ($P<0.05$) were noted at 6 and 8 weeks of age. Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

5.4.1.3 F3 generation

There was no effect of mating cross on systolic blood pressure at 4 weeks of age in the F3 generation (Figure 5.3). This continued at 6 weeks of age (Figure 5.3).Values between mating cross groups were similar, as were blood pressure values between male and female animals. At 8 weeks of age (Figure 5.3) there remained no difference in blood pressure between mating crosses of the F3 generation, likewise blood pressure values between male and female animals was also similar.

Animals that were maintained on a postnatal HF diet had lower ($P<0.05$) systolic blood pressure at 4 weeks of age (approximately 10%) in comparison to animals maintained on a postnatal chow diet, irrespective of sex. By 6 weeks of age this effect was no longer present.

Figure 5.3 F3 generation systolic blood pressures at 4, 6 and 8 weeks of age.

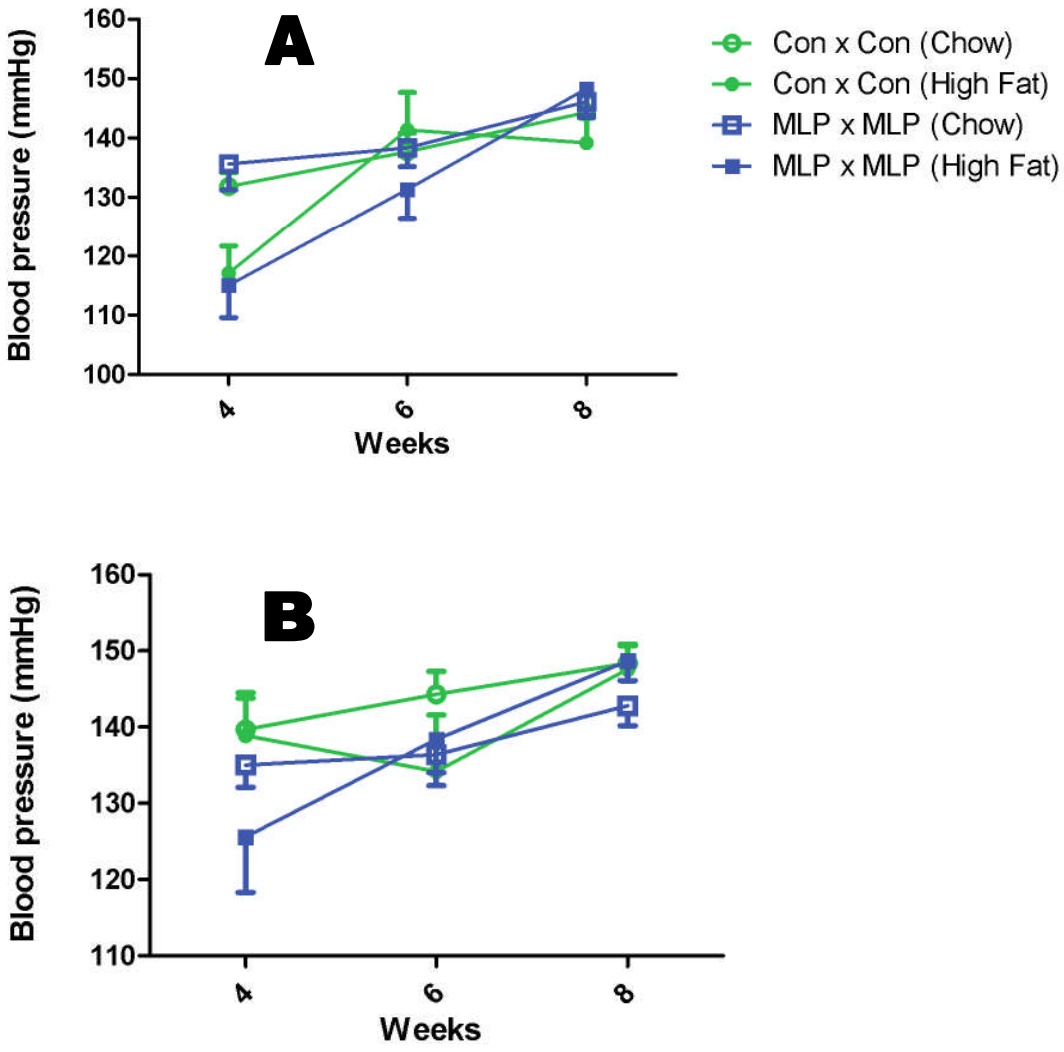


Figure 5.3 Data is shown as mean ± SEM. Graph A represents male systolic blood pressure at 4, 6 & 8 weeks of age. For Con x Con chow (n = 31); Con x Con HF (n = 11); MLP x MLP chow (n = 23); MLP x MLP HF (n = 8). Graph B represents female systolic blood pressure at 4, 6 & 8 weeks of age. For Con x Con chow (n = 29); Con x Con HF (n = 9); MLP x MLP chow (n = 24); MLP x MLP HF (n = 8). Significant effects of sex ($P<0.05$) and postnatal diet ($P<0.05$) were noted at 4 weeks of age. Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

Figure 5.4 8 week systolic blood pressures

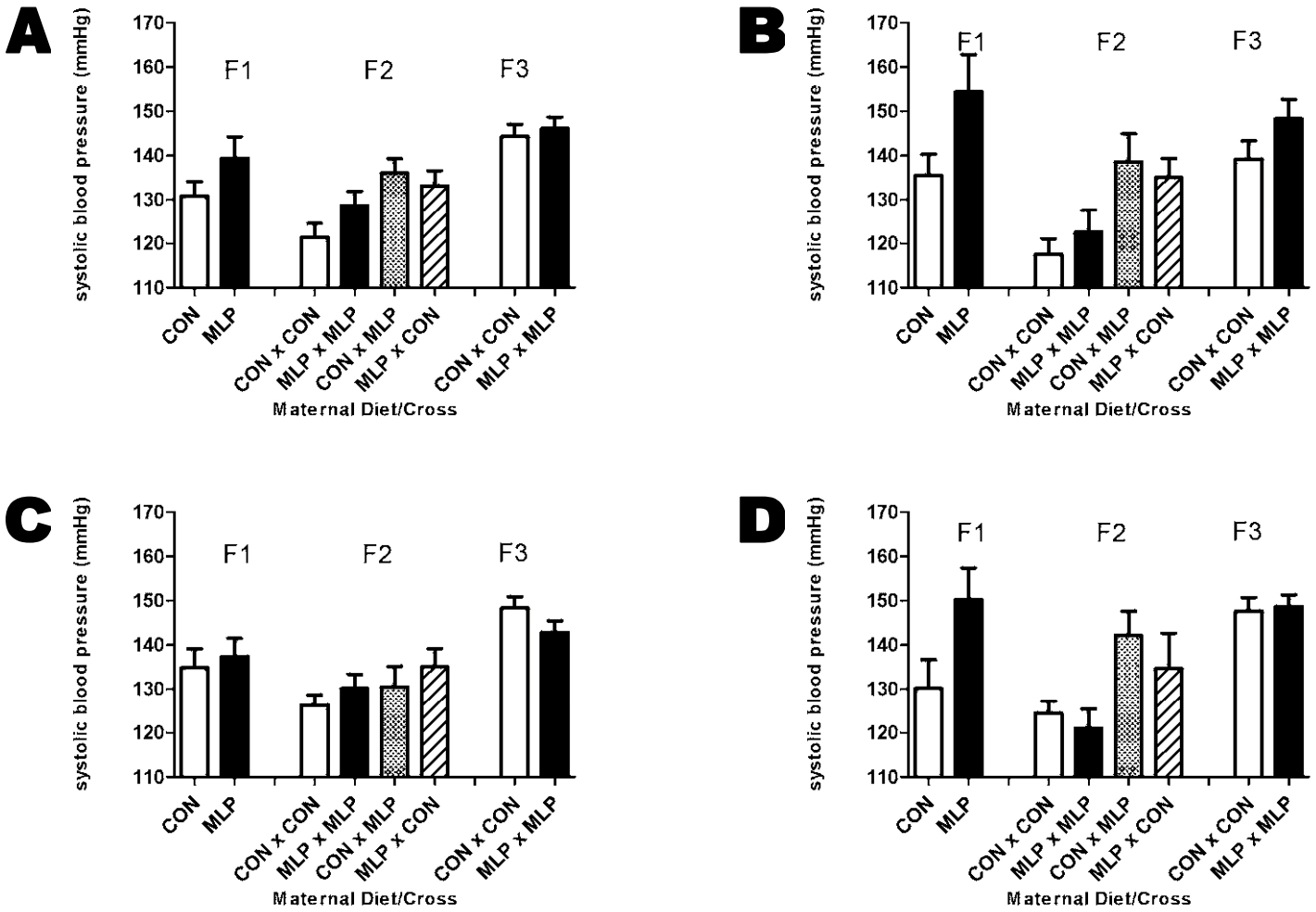


Figure 5.4 Data is shown as mean ± SEM. Graph A represents the systolic blood pressure at 8 weeks of age of male animals maintained on a chow postnatal diet. For F1 Con (n=31); MLP (n=28). F2 Con x Con (n=33); MLP x MLP (n=35); Con x MLP (n=24) and MLP x Con (n=23). F3 Con x Con (n=31) and MLP x MLP (n=23). Graph B represents the systolic blood pressure at 8 weeks of age of male animals maintained on a HF postnatal diet. For F1 Con (n=10); MLP (n=10). F2 Con x Con (n=11); MLP x MLP (n=10); Con x MLP (n=8) and MLP x Con (n=7). F3 Con x Con (n=11) and MLP x MLP (n=8). Graph C represents the systolic blood pressure at 8 weeks of age of female animals maintained on a chow postnatal diet. For F1 Con (n=29); MLP (n=29). F2 Con x Con (n=33); MLP x MLP (n=29); Con x MLP (n=24) and MLP x Con (n=26). F3 Con x Con (n=29) and MLP x MLP (n=24). Graph D represents the systolic blood pressure at 8 weeks of age of female animals maintained on a HF postnatal diet. For F1 Con (n=10); MLP (n=11). F2 Con x Con (n=11); MLP x MLP (n=11); Con x MLP (n=8) and MLP x Con (n=8). F3 Con x Con (n=9) and MLP x MLP (n=8). Significant effects of mating cross ($P<0.001$) were noted in the F1 and F2 generations. Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

5.4.2 Nephron Number

Nephron number in the left kidney was determined after cull at 10 weeks of age. F1 generation nephron complement (Figure 5.5) was lower ($P<0.001$) by approximately 35% in animals exposed to a MLP diet *in utero*, compared to control animals. Nephron numbers in male and female animals were similar. In the F2 generation (Figure 5.5), nephron complement followed the same trend as the F1 generation. Nephron numbers were approximately 40% lower ($P<0.001$) in all MLP mating crosses compared to controls (Con x Con). Nephron numbers in male and female animals remained similar. In the F3 generation (Figure 5.5) nephron complement was similar in the two groups. No difference in male and female animals was detected. Due to time constraints, the possible effects of postnatal HF feeding were not analysed within this investigation.

Figure 5.5 Nephron complement at 10 weeks of age.

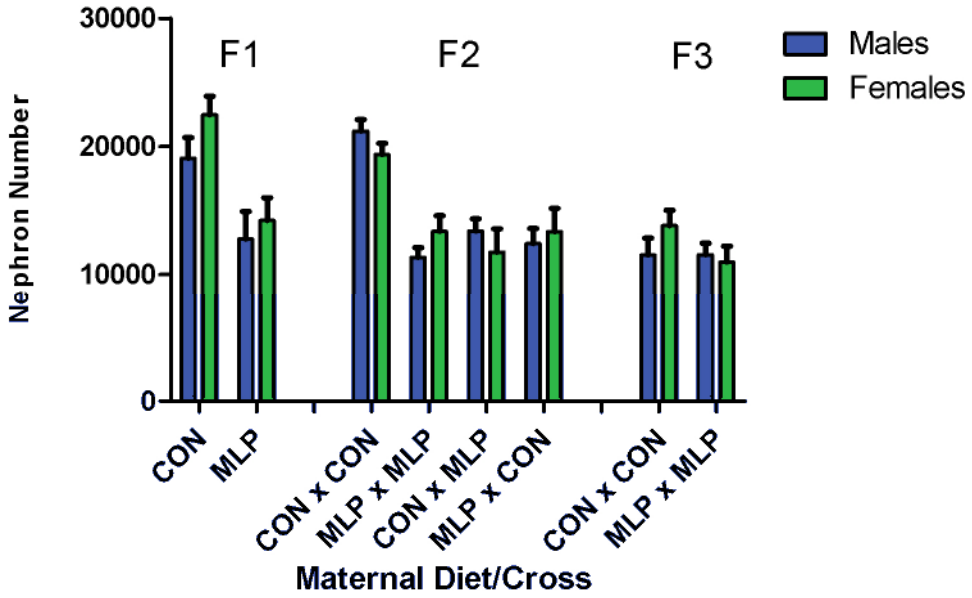


Figure 5.5 Data is shown as mean ± SEM, For F1 Con male (n=8); female (n=9), MLP male (n=11); female (n=9). F2 Con x Con male (n=22); female (n=22), MLP x MLP male (n=23); female (n=18), Con x MLP male (n=16); female (n=16), MLP x Con male (n=13); female (n=18). F3 Con x Con male (n=20); female (n=18), MLP x MLP male (n=14); female (n=16). Significant effects of maternal diet (F1: $P<0.001$) and mating cross (F2: $P<0.001$) were noted. Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*.

5.5 Discussion

5.5.1 Summary of findings

The links between prenatal exposure to maternal protein restriction and elevations in systolic blood pressure in the F1 generation are well established (Langley-Evans, 1996, 1997; Langley-Evans *et al.*, 1996b; Langley-Evans *et al.*, 2003; Langley-Evans *et al.*, 1994; Langley-Evans *et al.*, 1999a; Langley-Evans *et al.*, 1999b; Langley-Evans *et al.*, 1996d). The results within this chapter show that there is clear transgenerational transmission of this effect to the F2 generation.

Table 5.1 Summary of findings.

	Generation		
	F1	**F2**	**F3**
4 Week blood pressure	Decreased in MLP maternal diet group, decreased with HF postnatal feeding	No change with mating cross or postnatal diet	No change with mating cross, decreased with HF postnatal feeding
6 Week blood pressure	No change with maternal diet or postnatal diet	Increased in all MLP mating cross groups, no change with postnatal diet	No change with mating cross or postnatal diet
8 Week blood pressure	Increased in MLP maternal diet group, no change with postnatal diet	Increased in all MLP mating cross groups, no change with postnatal diet	No change with mating cross or postnatal diet
Nephron complement	Decreased in MLP maternal diet group	Decreased in MLP mating cross groups	No change with mating cross

Table 5.1 shows a brief summary of the findings from the assessment of systolic blood pressure and nephron complement in the F1, F2 and F3 generations.

Table 5.1 shows a brief summary of the findings from the blood pressure and nephron number analyses of the animals within this investigation. Blood pressure within the F1 and F2 generations by 8 weeks of age was higher in animals exposed to MLP diet, either *in utero* or by mating cross. This increased blood pressure went hand in hand with a reduction in nephron number in the MLP groups of both generations. There was no effect of maternal diet/ mating cross on the blood pressure or nephron complement of the F3 generation. There was no correlation between blood pressure at 4, 6 or 8 weeks of age with nephron number at 10 weeks of age in any generation.

5.5.2 Effect of prenatal protein restriction and mating cross

Data within the present chapter considered the impact of prenatal protein restriction upon blood pressure and renal development. Previous work within our laboratory has characterised the programming effects of a MLP diet during rat pregnancy upon blood pressure (Langley-Evans, 1996; Langley-Evans *et al.*, 1996b; Langley-Evans *et al.*, 1994; Langley-Evans *et al.*, 1999b; Langley-Evans *et al.*, 1996d) and renal development and function (Langley-Evans *et al.*, 2003; Langley-Evans *et al.*, 1999a) in the F1 generation. Data from the current investigation has for the first time shown that the effects of protein restriction *in utero* may not be limited to the first generation. The present investigation combines measurements of blood pressure with analysis of nephron complement to show that a relatively mild protein restriction during gestation has far-reaching implications for future generations. Much of the work previously undertaken on transgenerational programming has

focussed purely on a mechanistic viewpoint, without extensive effort to demonstrate that there is intergenerational transmission of any phenotype (Anway *et al.*, 2008).

The method utilised within this investigation to analyse blood pressure was the indirect tail cuff method (Sherman and Langley-Evans, 1998). Currently there is much debate over which is the most appropriate method to use in order to monitor blood pressure. Kurtz and colleagues (2005) recently published a review containing a list of recommendations for blood pressure measurement in animals; this review lists both the advantages and disadvantages of both direct and indirect methods of blood pressure monitoring and concluded that the method utilised depends upon the objectives of the investigation (Kurtz *et al*., 2005). Although current opinion within the scientific community is leaning towards the use of radio-telemetry systems similar to the equipment used in the study by Tonkiss and colleagues (1998), this procedure was unfeasible in this investigation due to the large number of animals that needed monitoring, in addition the review by Kurtz and colleagues (2005) specifically states that tail cuff is appropriate for large studies in which the aim is to identify large differences in blood pressure between groups.

It should be noted that a host of other external factors are able to affect blood pressure including ambient room temperature, light cycle, noise levels, duration of human contact, number of animals per caging unit, proximity to other animals undergoing experimental procedures, cage unit size and design, and access to supplemental items such as toys, treadmills, and hiding spaces within the cage unit. Therefore, it is important to keep in mind that many environmental factors can have substantial effects on cardiovascular function and blood pressure monitoring, therefore all factors should also be considered when using either indirect or direct methods for measuring blood pressure (Kurtz *et al*., 2005).

The tail-cuff method used within this study analyses blood pressure without the use of heating, therefore preventing heat-stress of the animals that is usually associated with the tail cuff procedure (Kubota *et al.*, 2006; Tonkiss *et al*., 1998). The tail cuff method has been expertly utilised in several previous studies within our own and other research groups (Langley-Evans *et al.*, 1996b; Langley-Evans and Jackson, 1995, 1996). Recent research has indicated that the rise in blood pressure associated with restraint stress (measured by telemetry) is similar in control and MLP offspring (Swali *et al.*, unpublished data) conflicting with evidence reported by Tonkiss and colleagues (1998). Previous studies have concluded that the tail-cuff method is a sensitive and accurate technique for determining blood pressure in conscious rats (Ibrahim *et al.*, 2006) and it has demonstrated that similar measurements are obtained by both the radio telemetry and the unheated tail-cuff methods (Kubota *et al.*, 2006).

The MLP diet protocol used within this investigation is the best characterised and most widely studied model of nutritional programming. As previously stated, the exposure to a MLP diet *in utero* has been shown to consistently induce persistent high blood pressure in the offspring (Langley and Jackson, 1994). Earlier studies have indicated that MLP-exposed offspring may be hypertensive from as early as weaning (Langley-Evans *et al.*, 1994) and that this persists throughout adult life (Langley-Evans and Jackson, 1995). As expected, systolic blood pressure was higher in MLP exposed animals of the F1 generation. However, within this study, no evidence of raised blood pressure was observed until 8 weeks of age. In fact a lower blood pressure was noted in MLP offspring of the F1 generation at 4 weeks of age. Although atypical of previous research on the MLP diet, similar outcomes have been observed following the maternal restriction of iron (Crowe *et al.*, 1995; Gambling *et*

al., 2003), where high blood pressure follows a period of lower blood pressure around the time of weaning. The current investigation is the first study to examine the interplay between blood pressure and renal structure across 3 successive generations. It has demonstrated that the effects of a mild protein restriction and its resultant phenotype are passed from the F1 generation to the F2 generation via both the paternal and maternal lines (as all F2 crosses involving low protein resulted in raised systolic blood pressure). The transmission of this phenotype must therefore involve epigenetic modifications, specifically in the gametes.

The kidney plays a major role in homeostatic regulation of blood pressure and, in rats, fetal exposure to the low protein diet has been shown to reduce nephron complement by up to 30% (Langley-Evans *et al.*, 1999a; Welham *et al.*, 2002). The present study shows that this reduction in nephron number is passed to a second generation via both parental lines. It is hypothesised that to compensate for nephron deficits at birth and to maintain renal haemodynamic functions, blood pressure within the nephrons is increased to maintain glomerular perfusion. Thus a cycle of progressive nephron loss begins whereby increasing blood pressure results in the loss of more nephrons eventually resulting in hypertension (Mackenzie, 1995). There is, however, emerging evidence that the nutritional programming nephron number and of blood pressure are independent processes occuring in a sex specific manner (McMullen and Langley-Evans, 2005). The relationship between nephron number and subsequent blood pressure is not clear-cut as there is evidence that hypertension can occur without a reduction in nephron number (Jackson *et al.*, 2002). Live human kidney donation studies support this, as they show that uninephrectomy in adult renal transplant donors carries a very low risk of hypertension (Gossmann *et al.*, 2005). It has been hypothesised that this difference maybe due primarily to age, for example

the effect of fewer nephrons from birth may be different from the loss of nephrons in the mature individual. Data from animal studies support this age-dependence hypothesis (Moritz *et al.*, 2002; Woods, 1999). It is thought that the differing intensity of the renal adaption to reduced nephron number may be key to this phenomenon. The hyperfiltration response to reduced nephron number is more effective in the young kidney (Chou and Hsu, 1991). A more effective response to preserve GFR in the short-term may therefore enhance hypertension in the long term. Although there is much debate over the relationship between nephron number and blood pressure it is clear from previous studies and the data within this chapter that the restriction of protein during gestation has the potential to generate a phenotype that features both reduced nephron complement and hypertension. It is apparent that this phenotype is subsequently passed on to a second generation via both parental lines.

Torrens *et al.*, (2008) have also recently shown that high blood pressure can be transmitted to the F2 generation following maternal protein restriction, however this study was only powered to assess maternal transmission. Within the study by Torrens and colleagues, there was evidence of an impaired vasodilatory response to acetylcholine, suggesting that in addition to renal programming, endothelial dysfunction may contribute to the transgenerational phenomena.

An interesting and unexpected result to arise from the current investigation was that the blood pressures of control animals in F3 were elevated, and nephron counts decreased, compared to the F1 groups. The reason for this is unknown. It is however possible that these intergenerational differences may effectively be due to in-breeding of a small colony in the F2 and F3 generations. Furthermore, the fact that the present study shows that the experience of the mother and grandmother of each

animal determines blood pressure, and nephron complement may be of relevance. The grandmaternal influence on the F1 generation will have been different to the grandmaternal influence on the F3 generation, and it is this difference which could explain the changes.

It is evident from the data on blood pressure and nephron number presented in this chapter, that the offspring (F2) of either male or female rats exposed to MLP diets *in utero* (F1) develop the same traits as their parents. Other research groups have also found evidence of transgenerational programming (Beach, 1982; Waterland *et al.*, 2007; Zambrano *et al.*, 2005), however, like the study by Torrens and colleagues, they have only considered the maternal transmission of phenotypes. These studies used stud males that were unexposed to dietary and hormonal challenges *in utero* to breed from programmed females. One of the main problems with such studies is that they cannot conclusively demonstrate that maternal traits are transmitted to the next generation via programmed influences on the ovum, as changes to the maternal environment and the composition of proteins in the embryo may play a key role. Metabolic traits such as glucose intolerance or cardiovascular traits such as raised blood pressure could be programmed as a response to the prevailing maternal environment during F1 pregnancy. For example, it has been previously shown that MLP diets in rat pregnancy and lactation lead to glucose intolerance. F2 offspring derived from glucose intolerant females also show this trait, which is acquired through glucose spill-over across the placenta (Reusens and Remacle, 2001).

A major strength of the research design of the present study was the use of males from the F1 and F2 generations in the mating crosses to produce the successive generations. This allows us to assess the potential contribution of the

paternal, as well as maternal factors, in transgenerational programming. In the present study we can at least conclude that male animals must transmit a programming signal to their offspring via alterations to the sperm genome. Arguments suggesting that maternal blood pressure tracking (familial aggregation) may produce these effects (Feng *et al.*, 2008; Hunt *et al.*, 2000; Liese *et al.*, 1997), fall short of the mark as high blood pressure was passed down both the maternal and paternal lines. This argument also cannot explain the decrease in nephron number found in both the F1 and F2 populations. It could be hypothesised that there may be the potential for some form of selection, whereby only fetuses with elevated blood pressures survive the pregnancy insult. This would potentially select for a hypertensive genotype. This "survivor" effect is not plausible, however, within this study as pregnancy outcomes were similar in rats fed to control and MLP diets.

5.5.3 Postnatal high fat feeding

There was little impact of postnatal HF feeding upon systolic blood pressure at any of the time points monitored. Reductions in blood pressure were indicated at 4 weeks of age in the F1 and F3 generations. Lard is comprised of 38 - 43% saturated fats and 56 - 62% unsaturated fats, of which around 50% are monounsaturated fatty acids (MUFA). Previous research has indicated that the feeding of lard-based high fat diet both pre- and postnatally significantly increase systolic blood pressure (Kaufman *et al.*, 1991; Khan *et al.*, 2003; Yoshioka *et al.*, 2000). The reason why there was no observed change in systolic blood pressure in the animals maintained on a high fat postnatal diet within the current investigation is unknown. In a comparison of the diets utilised by both the current investigation and the study by Yoshioka and

colleagues (2000) both diets contained the same amount of lard (29%). One possible explanation is that the standard chow diet utilised within the current investigation is masking any effects of the lard based diet. The standard chow diet used within this study has higher concentrations of polyunsaturated fatty acids (PUFA), especially n-6, than are present in lard. Previous studies have indicated that n-6 fatty acids have a hypertensive effect (Langley-Evans *et al.*, 1996a). Therefore, effectively both standard chow and high fat animals could have higher systolic blood pressure relative to a low n-6, low saturated fat diet.

5.5.4 Conclusions

The data presented within this chapter demonstrates that the feeding of a MLP diet *in utero* resulted in a phenotype comprising raised systolic blood pressure and reduced nephron complement. This was transmitted to a second generation via both the maternal and paternal lines. Although there is much debate over the link between nephron number and blood pressure it is important to note that nephron number is an important determinant of hypertension and renal disease (Hoy *et al.*, 2008). Further work will take a mechanistic viewpoint and attempt to identify possible gene targets involved in endothelial cell biology. Possible epigenetic modes of inheritance will be analysed in order to understand the molecular basis of the observed phenotype.

6.0 Prenatal protein restriction and the molecular basis of hypertension

6.1 Introduction

As described previously (section 1.2), it is clear that the risk of developing CVD and diabetes emerges as a consequence of interplay between genetic and environmental factors. Studies reported earlier in this thesis have indicated that the feeding of a MLP diet throughout gestation programmes a phenotype that consists of high systolic blood pressure combined with a lower complement of nephrons, which is passed from the F1 generation to the F2 generation. It has been postulated that undernutrition during gestation programmes long-term changes in gene expression, which alter metabolism in the developing fetus and result in cardiovascular abnormalities in later life (Barker *et al.*, 1993). Indeed the expression of genes that either protect against, or predispose to these conditions will be further modified by interactions between the genotype, early life nutrition, and the postnatal environment (Langley-Evans, 2006). A number of studies have indicated that prenatal undernutrition has the capacity to modulate the epigenetic regulation of gene expression (Burdge *et al.*, 2007a; Jaenisch and Bird, 2003). This raises the prospect that undernutrition can establish heritable changes to the epigenome and, as such, the disease programming effects of undernutrition in the fetal period may not be limited to the first generation. Data from within this thesis and emerging evidence from human and animal studies suggests that transgenerational effects may occur, whereby the consequences of deficits in maternal nutrition are subsequently passed on to the grand children (Beach, 1982; James, 2002; Pembrey, 1996). Indeed epigenetic changes within tissues and especially gametes could play a vital role in the transgenerational effect.

6.2 Objectives

The primary aim of the studies within this chapter was to assess changes in renal gene expression. Targeted microarray technology was used in order to assess potential gene targets, alongside real time PCR to determine the magnitude of the programmed changes between control and MLP exposed animals. The specific aims of this chapter were;

- To identify potential gene targets for qRT-PCR analysis using a microarray approach.
- To assess the impact of prenatal MLP exposure upon renal gene expression.
- Examine potential differences in the extent of DNA methylation between control and MLP exposed offspring.

6.3 Materials and methods

6.3.1 Microarray: determination of gene expression

A pathway focussed DNA microarray study was used in order to compare the expression of 113 genes in the kidney associated with endothelial cell biology, including genes involved in permissibility and vessel tone, angiogenesis, endothelial cell activation and endothelial cell injury. This technique was used as a hypothesis generating tool to assess appropriate gene targets to follow up in more detail via qRT-PCR (Zhang *et al.*, 2002).

Kidneys (n = 4 per group, selected for extremes of blood pressure) were removed at time of cull (10 weeks of age), snap frozen in liquid nitrogen and stored at -80°C prior to analysis (section 2.3). Total RNA was isolated using the Trizol

method (section 2.6.1.1) and integrity was determined using the Nanodrop® spectrophotometer (section 2.6.1.3). Microarray assays were performed as described in section 2.6. Images were analysed using a specially designed web-based analysis suite. Upon analysis of microarray data, a selection criterion of a two-fold increase or decrease in gene expression was applied.

6.3.2 qRT-PCR

RT-PCR (section 2.8) was used to detect the expression of mRNA for Matrix metallopeptidase 9 (MMP9), Granzyme B (GZMB), Endothelial cell growth factor 1 (ECGF1), Endothelin receptor type A (EDNRA) and Insulin-like growth factor 2 (IGF2) mRNA in rat kidneys (n = 6 per group). Expression was determined as described in section 2.8.

6.3.3 Cytosine extension assay

The cytosine extension assay was used as a method for analysing global DNA methylation (n = 6 per group). The assay was performed as described in section 2.7.2.

6.3.4 Statistical Analysis

All data was analysed using the Statistical Package for Social Sciences (SPSS, Inc, Chicago, IL, Version 14.0). Differences between groups were assessed using a mixed model ANOVA (fixed factors, maternal diet, sex and age). Values are expressed as mean ± S.E.M. $P<0.05$ was considered as significant.

6.4 Results

6.4.1 Gene expression

6.4.1.1 DNA Microarray Studies

In the F1 generation a total of 38 genes out of 113 were up-regulated two-fold or greater, and 12 genes were down-regulated by at least 50% (Table 6.1). However, none of these changes were significant when subjected to statistical analysis using a mixed model ANOVA. A total of 7 genes were two-fold up-regulated and 30 genes were 50% down-regulated (Table 6.2) within the F2 generation. Similar to the F1 generation, none of these changes were statistically significant. Within the F3 generation, 3 genes were up-regulated two-fold or more, and 30 genes were more than 50% down-regulated (Table 6.3). Although some significant changes in gene expression were seen within the F3 generation, these genes were considered to be irrelevant to the hypothesis, as they were unchanged within the F1 and F2 generations.

The microarray data suggested that there was a relatively narrow profile of genes that were programmed by fetal exposure to MLP diet *in utero* that were comparable across all the generations. Using the selection criteria described in section 2.6.2.8, a total of 11 genes were highlighted as being differently expressed in rat kidneys of MLP-exposed animals compared to control animals. Out of the 113 genes analysed 4 common genes were found to be up-regulated two-fold or more within the F1 and F2 generation (Figure 6.1). These were Cxcl2, Cxcl11, Ecgf1 and Gzmb. Both Cxcl2 and Cxcl11 were disregarded as these are chemokines secreted by macrophages in response to stress or injury and therefore were not thought to be key in the programming of the kidneys *in utero* but were elevated due to postnatal

stimuli. 2 common genes were found to be down-regulated 50% or more within the F1 and F2 generations (Figure 6.2), these were Ednra and Il7, both were selected for further study. A further 5 genes were found to be down-regulated by 50% or more in all 3 generations (Figure 6.2). From these five genes Mmp9 was selected for further analysis as previous research has suggested it has a role within uteric bud branching in the kidneys (Lelongt and Ronco, 2002).

Due to expense and time constraints a decision was made to analyse five of the genes identified by the microarray analysis by real time PCR (2 genes up-regulated and 3 genes down-regulated), these genes were Matrix metallopeptidase 9 (MMP9), Granzyme B (GZMB), Endothelial cell growth factor 1 (ECGF1), Endothelin receptor type A (EDNRA) and Interlukin 7 (Il7).

Table 6.1 Comparrison of gene expression in kidneys of control and MLP male animals of the F1 generation.

Gene	Maternal Diet		Fold Change	P Value
	Control	MLP		
Ifnb1	.0050	.0367	7.3400	.4795
Edn1	.0075	.0433	5.7733	.4965
Il11	.0050	.0267	5.3400	.5035
Itgav_predicted	.0050	.0267	5.3400	.5035
Agtr2	.0125	.0667	5.3360	.4427
Il3	.0025	.0133	5.3200	.5035
Pdgfra	.0025	.0133	5.3200	.5035
Bcl2l1	.0125	.0633	5.0640	.3747
Edn2	.0075	.0367	4.8933	.5118
Angpt2	.0150	.0733	4.8867	.3976
Agt	.0150	.0733	4.8867	.2468
Birc4	.0150	.0700	4.6667	.4592
Angpt1	.0175	.0800	4.5714	.4426
Bcl2	.0150	.0667	4.4467	.2795
Ace2	.0200	.0867	4.3350	.4301
Birc1b	.0125	.0500	4.0000	.4951
Edn3_mapped	.0075	.0300	4.0000	.5350
Fgf2	.0100	.0400	4.0000	.5350
Flt1	.0075	.0300	4.0000	.5350
Mmp1a_predicted	.0050	.0200	4.0000	.5350
Ccl20	.0150	.0567	3.7800	.5079
Chga	.0125	.0433	3.4640	.3576
Ccl2	.0200	.0667	3.3350	.5009
Blr1	.0175	.0567	3.2400	.3528
Cxcl1	.0150	.0433	2.8867	.3006
Cxcl11	.0100	.0267	2.6700	.6004
Cxcl2	.0100	.0267	2.6700	.6004
Flt3	.0100	.0267	2.6700	.5385
Ecgf1	.0075	.0200	2.6667	.5994
Ccl5	.0200	.0533	2.6650	.4450
Cpb2	.0125	.0333	2.6640	.6011
Alox5	.0350	.0900	2.5714	.3630
Agtr1b	.0325	.0767	2.3600	.5279
Csf3	.0200	.0467	2.3350	.4838
Flt4	.0100	.0233	2.3300	.5845
Vwf	.0325	.0733	2.2554	.3510
Gzmb	.0075	.0167	2.2267	.6431
Fgf1	.0150	.0300	2.0000	.5580
Bcl2a1	.0350	.0667	1.9057	.4590
Birc3	.1000	.1900	1.9000	.1386
Birc5	.0450	.0833	1.8511	.4096
Csf2	.0275	.0500	1.8182	.6730
Casp8	.0250	.0433	1.7320	.5871
Cdh5_predicted	.0250	.0433	1.7320	.5913
AS1R2	.0175	.0300	1.7143	.3844
Enpep	.0125	.0200	1.6000	.7085
Itgb3	.0125	.0200	1.6000	.7506
Cflar	.0350	.0533	1.5229	.5993
Mmp2	.0100	.0133	1.3300	.8457
Tgfb1	.0025	.0033	1.3200	.8457
BAS2C	.8925	1.1600	1.2997	.3390

BAS2C	.9825	1.2233	1.2451	.4105
Casp3	.0625	.0767	1.2272	.7544
Itgb1	.0800	.0900	1.1250	.8155
Gapdh	.9775	1.0600	1.0844	.3863
Gapdh	.9800	1.0600	1.0816	.3981
Casp6	.0550	.0567	1.0309	.9701
Ace	.2150	.2133	.9921	.9836
Tek	.0750	.0733	.9773	.9743
Cx3cl1	.6300	.5667	.8995	.7983
Casp1	.1300	.1167	.8977	.8404
Plg	.0600	.0533	.8883	.8648
Icam2	.1800	.1567	.8706	.7995
Nos3	.4575	.3933	.8597	.7500
Sod1	1.0050	.8633	.8590	.3946
Ppia	1.0400	.8800	.8462	.3981
Adam17	.2400	.1933	.8054	.6841
Rpl32	.9600	.7633	.7951	.4418
Npr1	.4675	.3700	.7914	.5740
Anxa5	.2200	.1700	.7727	.6899
Ldha	.7825	.6000	.7668	.5939
Tnfsf10	.5950	.4533	.7618	.6010
Itga5	.3925	.2967	.7559	.5901
Mmp14	.7175	.5400	.7526	.5723
Plau	.5825	.4333	.7439	.5923
Kdr	.8100	.5800	.7160	.4330
Ednrb	.1075	.0767	.7135	.5993
Aldoa	.9350	.6600	.7059	.4626
Aldoa	.9475	.6633	.7001	.4521
Cradd	.1975	.1367	.6922	.6310
Timp1	.4025	.2700	.6708	.4700
Col18a1	.3200	.2133	.6666	.4621
Tfpi2	.0050	.0033	.6600	.8090
Bax	.2325	.1433	.6163	.5342
Xdh	.0225	.0133	.5911	.7010
Icam1	.0750	.0433	.5773	.4827
Il6	.0125	.0067	.5360	.6018
Tfpi	.0925	.0467	.5049	.2978
Fn1	.4100	.1933	.4715	.2996
Il15	.2250	.1000	.4444	.3199
Pla2g4c	.0450	.0167	.3711	.3782
Il1b	.0475	.0167	.3516	.3786
F3	.1075	.0367	.3414	.1902
Plat	.3325	.1133	.3408	.2043
Ednra	.0800	.0267	.3338	.4190
Pdgfrb	.0300	.0100	.3333	.5599
Il7	.0250	.0067	.2680	.2963
Mmp9	.1075	.0233	.2167	.2124
Nos2	.0725	.0133	.1834	.1364

Table 6.1 shows gene expression in the F1 generation. Fold difference indicates a ratio of expression for MLP Vs Control. Genes exhibiting a two-fold up-regulation are highlighted in blue and genes exhibiting less than 50% of control expression are shown in red. For a full list of gene names please see appendix. No significant effects were noted. Data analysed using a mixed model ANOVA.

Table 6.2 Comparrison of gene expression in kidneys of control and MLP mating cross male animals of the F2 generation.

Gene	Breeding Cross		Fold Change	Cross	Fold Change	Cross	Fold Change	P Value
	Con X Con	MLP X MLP		MLP X Con		Con X MLP		
Gzmb	.0025	.0125	5.0000	.0050	2.0000	.0100	4.0000	.6236
Il6	.0025	.0100	4.0000	.0025	1.0000	.0150	6.0000	.3904
Ecgf1	.0050	.0100	2.0000	.0075	1.5000	.0200	4.0000	.7196
AS1R1	.0025	.0050	2.0000	.0000	.0000	.0100	4.0000	.3763
Cxcl11	.0100	.0175	1.7500	.0125	1.2500	.0150	1.5000	.9512
Cxcl2	.0075	.0125	1.6667	.0100	1.3333	.0175	2.3333	.8895
Enpep	.0075	.0125	1.6667	.0075	1.0000	.0150	2.0000	.8477
Chga	.0175	.0275	1.5714	.0175	1.0000	.0200	1.1429	.9471
Flt3	.0050	.0075	1.5000	.0075	1.5000	.0075	1.5000	.9873
Bcl2	.0325	.0375	1.1538	.0350	1.0769	.0200	.6154	.8814
Cflar	.0350	.0400	1.1429	.0350	1.0000	.0250	.7143	.8999
Cxcl1	.0200	.0225	1.1250	.0150	.7500	.0150	.7500	.9226
Bcl2l1	.0250	.0275	1.1000	.0250	1.0000	.0225	.9000	.9965
Blr1	.0300	.0325	1.0833	.0250	.8333	.0250	.8333	.9834
Gapdh	.9875	1.0600	1.0734	.9875	1.0000	1.0050	1.0177	.3885
Gapdh	.9900	1.0625	1.0732	.9900	1.0000	1.0050	1.0152	.3700
BAS2C	1.0000	1.0600	1.0600	.9925	.9925	1.0200	1.0200	.5132
Rpl32	.9675	1.0050	1.0388	.9700	1.0026	.9675	1.0000	.6331
Sod1	1.0000	1.0350	1.0350	.9950	.9950	1.0025	1.0025	.5798
Nppb	.0025	.0025	1.0000	.0000	.0000	.0050	2.0000	.7256
Vcam1	.0050	.0050	1.0000	.0000	.0000	.0075	1.5000	.6621
BAS2C	.9550	.9500	.9948	.9500	.9948	.9475	.9921	.9997
Birc1b	.0225	.0200	.8889	.0175	.7778	.0225	1.0000	.9937
Serpine1	.0225	.0200	.8889	.0150	.6667	.0075	.3333	.8566
Angpt2	.0375	.0325	.8667	.0350	.9333	.0225	.6000	.9119
Ppia	1.0225	.8775	.8582	1.0225	1.0000	.9925	.9707	.3785
Aldoa	.9375	.7925	.8453	.9575	1.0213	.8700	.9280	.2100
Nos3	.5725	.4825	.8428	.5550	.9694	.4575	.7991	.8752
Ccl5	.0300	.0250	.8333	.0200	.6667	.0250	.8333	.9599
Agtr2	.0250	.0200	.8000	.0175	.7000	.0225	.9000	.9850
Edn3_map	.0125	.0100	.8000	.0125	1.0000	.0100	.8000	.9933
Ednrb	.0700	.0550	.7857	.0775	1.1071	.0275	.3929	.6812
Aldoa	.9250	.7200	.7784	.9500	1.0270	.8300	.8973	.2787
Cdh5_pre	.0425	.0325	.7647	.0275	.6471	.0300	.7059	.9024
Flt4	.0100	.0075	.7500	.0125	1.2500	.0075	.7500	.9637
Agtr1b	.0400	.0300	.7500	.0675	1.6875	.0250	.6250	.2729
Csf3	.0300	.0225	.7500	.0200	.6667	.0175	.5833	.8803
Col18a1	.2450	.1750	.7143	.2175	.8878	.2375	.9694	.9621
Bcl2a1	.0600	.0425	.7083	.0500	.8333	.0250	.4167	.4946
Agt	.0500	.0350	.7000	.0400	.8000	.0225	.4500	.6740
Ccl20	.0325	.0225	.6923	.0200	.6154	.0200	.6154	.8952
Birc4	.0400	.0275	.6875	.0250	.6250	.0225	.5625	.8287
Mmp14	.5425	.3650	.6728	.3450	.6359	.7575	1.3963	.3203
Alox5	.0650	.0425	.6538	.0550	.8462	.0550	.8462	.8277
Ccl2	.0350	.0225	.6429	.0325	.9286	.0250	.7143	.9092
Pla2g4c	.0625	.0400	.6400	.0375	.6000	.0175	.2800	.8285
Ldha	.6200	.3900	.6290	.5625	.9073	.6350	1.0242	.7668
AS1	.0200	.0125	.6250	.0325	1.6250	.0475	2.3750	.4017
Birc5	.0600	.0375	.6250	.0500	.8333	.0425	.7083	.6311
AS1R2	.0450	.0275	.6111	.0525	1.1667	.0550	1.2222	.8746

Cx3cl1	.6400	.3850	.6016	.4775	.7461	.4675	.7305	.7570
Angpt1	.0625	.0375	.6000	.0550	.8800	.0250	.4000	.2942
Plau	.5275	.3150	.5972	.2425	.4597	.3950	.7488	.7620
F3	.0675	.0400	.5926	.1200	1.7778	.0300	.4444	.7250
Casp1	.1500	.0875	.5833	.1375	.9167	.0675	.4500	.6061
Casp3	.0825	.0475	.5758	.0650	.7879	.0300	.3636	.3600
Casp8	.0525	.0300	.5714	.0275	.5238	.0275	.5238	.7693
Birc3	.1400	.0775	.5536	.1075	.7679	.1350	.9643	.5459
Fn1	.2825	.1525	.5398	.1625	.5752	.1375	.4867	.7917
Icam2	.2625	.1400	.5333	.2375	.9048	.1850	.7048	.6929
Timp1	.4100	.2150	.5244	.2600	.6341	.1725	.4207	.4812
Kdr	.8325	.4225	.5075	.7225	.8679	.5550	.6667	.2617
Tnfsf10	.4700	.2375	.5053	.4800	1.0213	.5125	1.0904	.4600
Fgf2	.0100	.0050	.5000	.0075	.7500	.0075	.7500	.9537
Ace2	.0550	.0275	.5000	.0325	.5909	.0250	.4545	.5785
Anxa5	.2025	.1000	.4938	.1700	.8395	.1525	.7531	.8661
Npr1	.5425	.2650	.4885	.4175	.7696	.3375	.6221	.5110
Ace	.3075	.1450	.4715	.2150	.6992	.2725	.8862	.5816
Tek	.1150	.0525	.4565	.0550	.4783	.1100	.9565	.7523
Csf2	.0275	.0125	.4545	.0225	.8182	.0225	.8182	.8475
Edn2	.0125	.0050	.4000	.0100	.8000	.0100	.8000	.9138
Il7	.0375	.0150	.4000	.0150	.4000	.0200	.5333	.5725
Plat	.2875	.1125	.3913	.2025	.7043	.1650	.5739	.8460
Cpb2	.0325	.0125	.3846	.0125	.3846	.0225	.6923	.7315
Fgf1	.0325	.0125	.3846	.0125	.3846	.0225	.6923	.6619
Icam1	.1125	.0425	.3778	.0500	.4444	.0225	.2000	.3504
Casp6	.0700	.0250	.3571	.0650	.9286	.0300	.4286	.5491
Il15	.1525	.0525	.3443	.0975	.6393	.0875	.5738	.6945
Adam17	.2700	.0925	.3426	.2125	.7870	.1850	.6852	.6081
Itgb3	.0075	.0025	.3333	.0050	.6667	.0075	1.0000	.8892
Nos2	.0775	.0225	.2903	.0700	.9032	.0250	.3226	.6957
Ednra	.0700	.0200	.2857	.0250	.3571	.0175	.2500	.5033
Itgb1	.1225	.0350	.2857	.0500	.4082	.0200	.1633	.0941
Tfpi	.1025	.0275	.2683	.0600	.5854	.0300	.2927	.5938
Tnf	.0100	.0025	.2500	.0000	.0000	.0075	.7500	.6007
Il1b	.0350	.0075	.2143	.0250	.7143	.0100	.2857	.2145
Itga5	.3250	.0675	.2077	.2400	.7385	.4250	1.3077	.2266
Edn1	.0125	.0025	.2000	.0100	.8000	.0100	.8000	.8057
Bax	.2750	.0550	.2000	.1575	.5727	.1750	.6364	.6437
Plg	.1025	.0200	.1951	.0650	.6341	.0200	.1951	.3600
Mmp9	.2600	.0475	.1827	.0775	.2981	.0975	.3750	.1221
Cradd	.2475	.0175	.0707	.1150	.4646	.1475	.5960	.5397
Vwf	.0925	.0050	.0541	.0450	.4865	.0600	.6486	.6111
Flt1	.0050	.0000	.0000	.0075	1.5000	.0075	1.5000	.7462
Xdh	.0475	.0000	.0000	.0300	.6316	.0150	.3158	.5586

Table 6.2 shows gene expression in the F2 generation. Fold difference indicates a ratio of expression for MLP Vs Control. Genes exhibiting a two-fold up-regulation are highlighted in blue and genes exhibiting less than 50% of control expression are shown in red. For a full list of gene names please see appendix. No significant effects were noted. Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, eg. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*.

Table 6.3 Comparrison of gene expression in kidneys of control and MLP mating cross male animals of the F3 generation.

Gene	Cross		Fold Change	P Value
	Con X Con	MLP X MLP		
AS1	.0075	.0333	4.4400	.1120
Plg	.0125	.0400	3.2000	.4655
Fgf1	.0150	.0333	2.2200	.3201
Gzmb	.0075	.0133	1.7733	.6018
Ace2	.0325	.0567	1.7446	.4632
Alox5	.0475	.0800	1.6842	.5062
Angpt1	.0325	.0533	1.6400	.5138
Birc3	.1125	.1733	1.5404	.5844
Ccl2	.0300	.0433	1.4433	.6137
Kit	.0050	.0067	1.3400	.8090
Ocln	.0050	.0067	1.3400	.8090
Birc4	.0300	.0400	1.3333	.7370
Il6	.0075	.0100	1.3333	.8144
Angpt2	.0325	.0433	1.3323	.7069
Ecgf1	.0100	.0133	1.3300	.8206
Agt	.0325	.0400	1.2308	.7824
Blr1	.0300	.0367	1.2233	.8121
Cxcl1	.0225	.0267	1.1867	.8536
Icam2	.1200	.1400	1.1667	.7152
Cxcl2	.0175	.0200	1.1429	.8888
Il7	.0175	.0200	1.1429	.8786
Bcl2	.0300	.0333	1.1100	.9019
Ccl20	.0275	.0300	1.0909	.9264
BAS2C	.9825	1.0300	1.0483	.3072
Gapdh	.9800	1.0267	1.0477	.3349
Gapdh	.9800	1.0267	1.0477	.3349
BAS2C	.8975	.9300	1.0362	.6456
Sod1	.9900	1.0200	1.0303	.2607
Icam1	.0425	.0433	1.0188	.9816
Cdh5_pre	.0300	.0300	1.0000	1.0000
Rpl32	.9850	.9800	.9949	.8564
Enpep	.0175	.0167	.9543	.9556
Cradd	.0500	.0467	.9340	.9420
Ppia	1.0400	.9467	.9103	.3349
Birc5	.0550	.0500	.9091	.9001
Cxcl5	.0075	.0067	.8933	.9397
Ccl5	.0300	.0267	.8900	.9067
Agtr2	.0225	.0200	.8889	.9110
Csf2	.0225	.0200	.8889	.9189
Cflar	.0375	.0333	.8880	.9034
Aldoa	.9500	.8133	.8561	.2273
Aldoa	.9350	.7933	.8484	.1273
Cpb2	.0200	.0167	.8350	.8575
Nos3	.4450	.3700	.8315	.7062
Chga	.0250	.0200	.8000	.8240
Agtr1b	.0350	.0267	.7629	.7632
AS1R2	.0350	.0267	.7629	.7433
Cxcl11	.0175	.0133	.7600	.8026
Bax	.0750	.0567	.7560	.7332
Birc1b	.0225	.0167	.7422	.7795

Bcl2l1	.0275	.0200	.7273	.7560
Casp8	.0275	.0200	.7273	.7560
Ace	.1775	.1267	.7138	.5140
Npr1	.4025	.2767	.6875	.4635
Ednra	.0250	.0167	.6680	.7481
Edn1	.0150	.0100	.6667	.8090
Edn2	.0150	.0100	.6667	.8090
Edn3_map	.0150	.0100	.6667	.8090
Timp1	.3825	.2467	.6450	.4730
Bcl2a1	.0450	.0267	.5933	.6270
Fgf2	.0175	.0100	.5714	.7085
Casp6	.0475	.0267	.5621	.6175
Serpine1	.0375	.0200	.5333	.5042
Kdr	.8000	.4200	.5250	.0367
Flt4	.0200	.0100	.5000	.6159
Csf3	.0475	.0233	.4905	.4008
Tek	.0825	.0400	.4848	.3847
Ednrb	.1150	.0533	.4635	.5551
Il1b	.0300	.0133	.4433	.4569
Il3	.0075	.0033	.4400	.6729
Casp1	.1300	.0567	.4362	.3442
Casp3	.0850	.0367	.4318	.2997
Tnfsf10	.5625	.2400	.4267	.0730
Plau	.5425	.2300	.4240	.3652
Il15	.1900	.0800	.4211	.4643
Ldha	.7625	.3133	.4109	.0370
Cx3cl1	.7075	.2833	.4004	.1571
F3	.0750	.0267	.3560	.4133
Col18a1	.2425	.0767	.3163	.2800
Itga5	.2850	.0900	.3158	.0111
Anxa5	.2475	.0700	.2828	.2315
Mmp9	.0950	.0267	.2811	.4038
Adam17	.3125	.0867	.2774	.2057
Flt1	.0125	.0033	.2640	.5691
Flt3	.0125	.0033	.2640	.5691
Itgb3	.0125	.0033	.2640	.5691
Fn1	.3650	.0933	.2556	.2426
Mmp14	.5850	.1467	.2508	.0067
Vwf	.0300	.0067	.2233	.2206
Itgb1	.0950	.0200	.2105	.2228
Plat	.2450	.0433	.1767	.2654
Pla2g4c	.1850	.0300	.1622	.3967
Nos2	.1425	.0167	.1172	.3450
Pdgfrb	.0650	.0067	.1031	.3692

Table 6.3 shows gene expression in the F3 generation. Fold difference indicates a ratio of expression for MLP Vs Control. Genes exhibiting a two-fold up-regulation are highlighted in blue and genes exhibiting less than 50% of control expression are shown in red. For a full list of gene names please see appendix. Significant effects were noted in genes Kdr, Ldha, Itga5 and Mmp14. Data analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, eg. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to MLP diet *in utero*.

6.4.1.2 Justification of gene choices

Figure 6.1 Genes that were up-regulated two-fold or more.

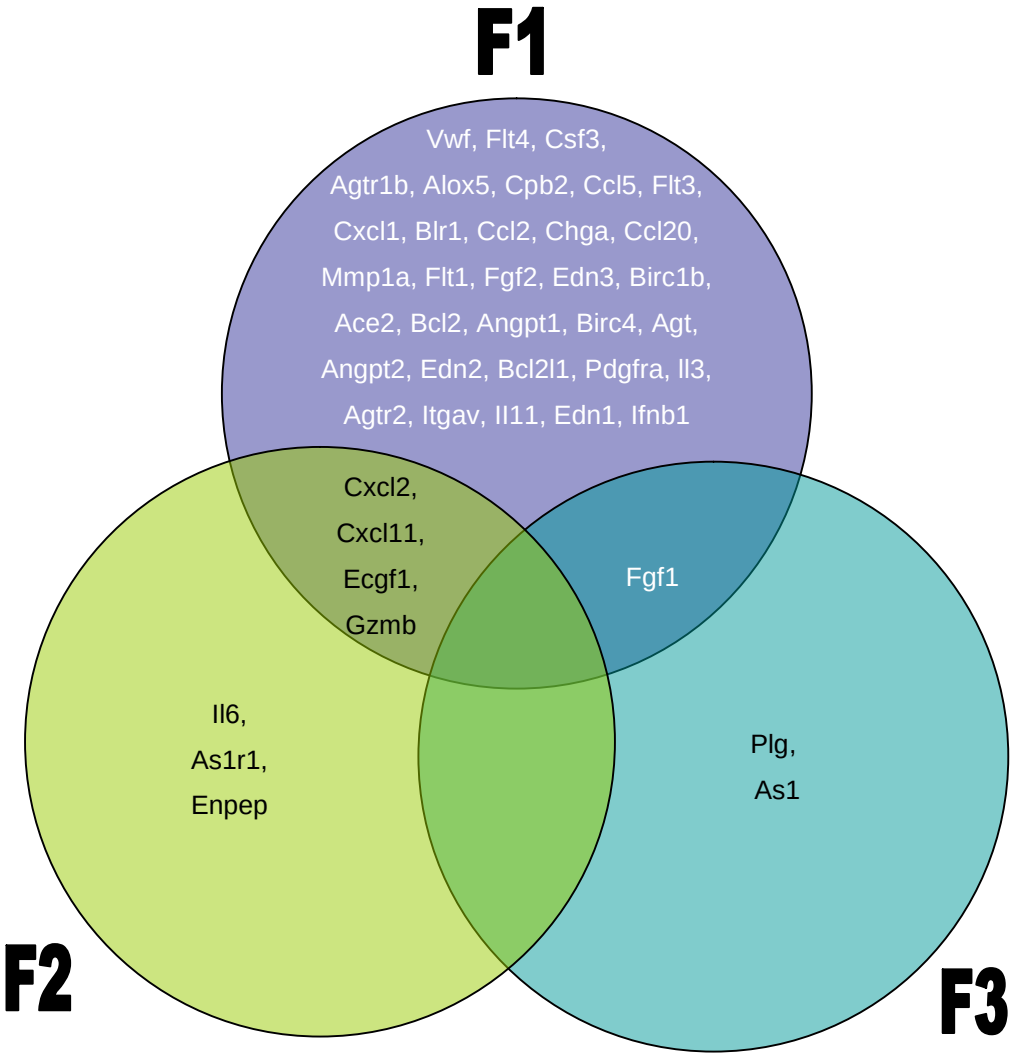


Figure 6.1 shows common gene expression between generations. Only genes expressing an up-regulation of two-fold or more are shown within this figure.

Figure 6.2 Genes that were down-regulated 50%.

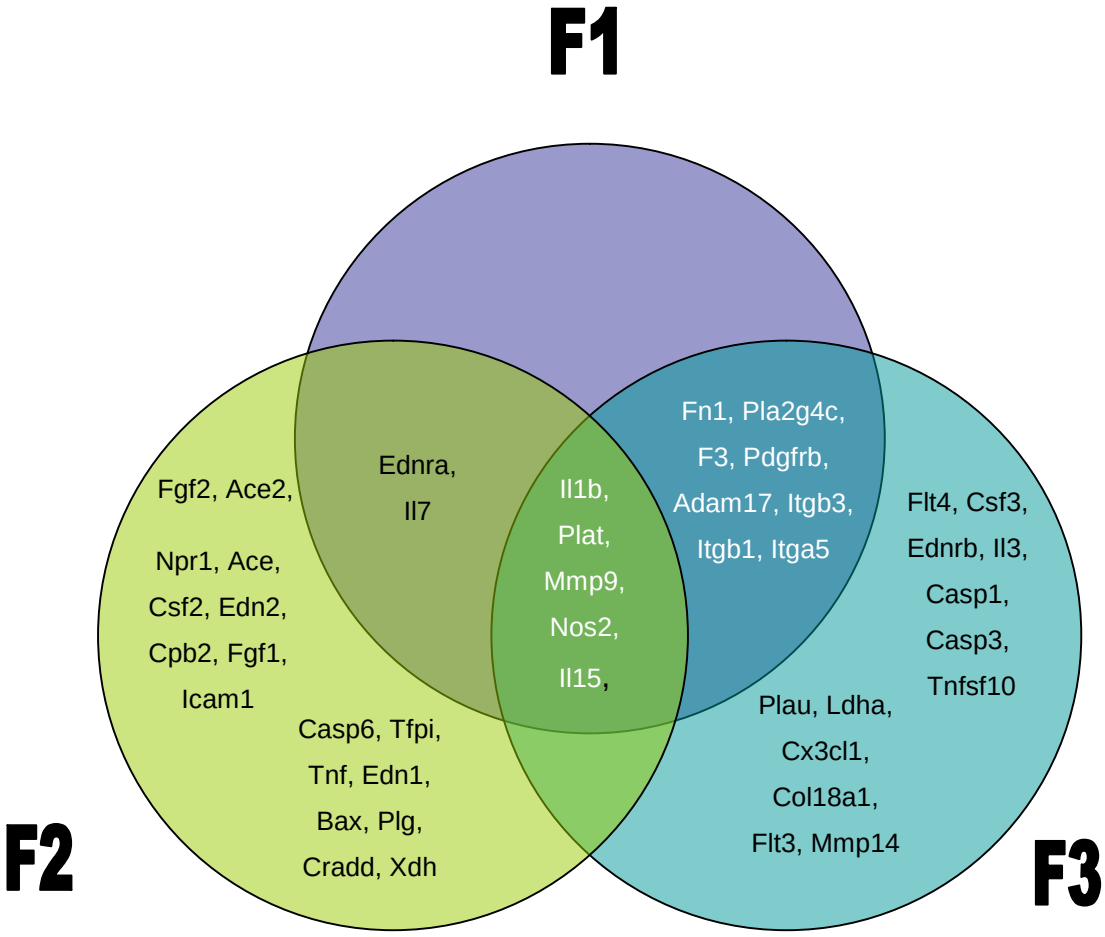


Figure 6.2 shows common gene expression between generations. Only genes expressing a down-regulation of 50% or more are shown within this figure.

6.4.1.3 qRT-PCR

As previously described (Section 6.3.2), microarray data analysis highlighted 5 gene targets for further study; Matrix metallopeptidase 9 (MMP9), Granzyme B (GZMB), Endothelial cell growth factor 1 (ECGF1), Endothelin receptor type A (EDNRA) and Interleukin 7 (Il7). However, due to the short gene sequence of Il7 (465bp) we were unable to design a working primer and probe set, therefore Il7 was disregarded. Further to this studies by Heijmans and colleagues (2008) have found evidence of hypomethylation of IGF-2 in their studies of the Dutch Hunger Winter

(Heijmans *et al.*, 2008). Therefore the decision was made to analyse the mRNA expression of IGF-2 within this study. The effect of postnatal HF feeding was not analysed within gene expression analysis. The housekeeper gene used within the real time PCR analysis was 364B.

6.4.1.3.1 364B expression (housekeeper gene)

364B expression within all 3 generations was similar. There was no effect of MLP exposure *in utero* or no effect of mating cross on expression (Figures 6.3, 6.4 & 6.5).

Figure 6.3 F1 generation 364B expression in 10 week old rat kidneys.

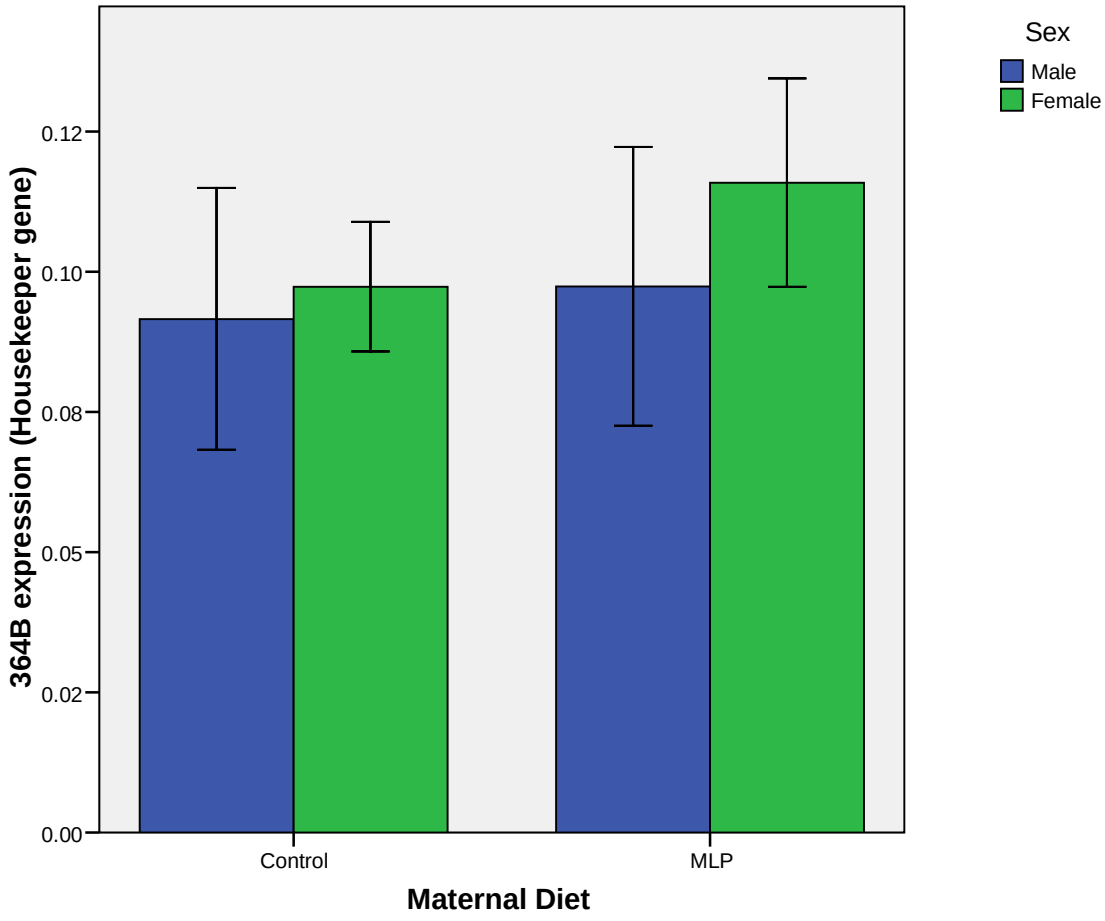


Figure 6.3 364B mRNA expression in 10 week old rat kidneys (F1). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA.

Figure 6.4 F2 generation 364B expression in 10 week old rat kidneys.

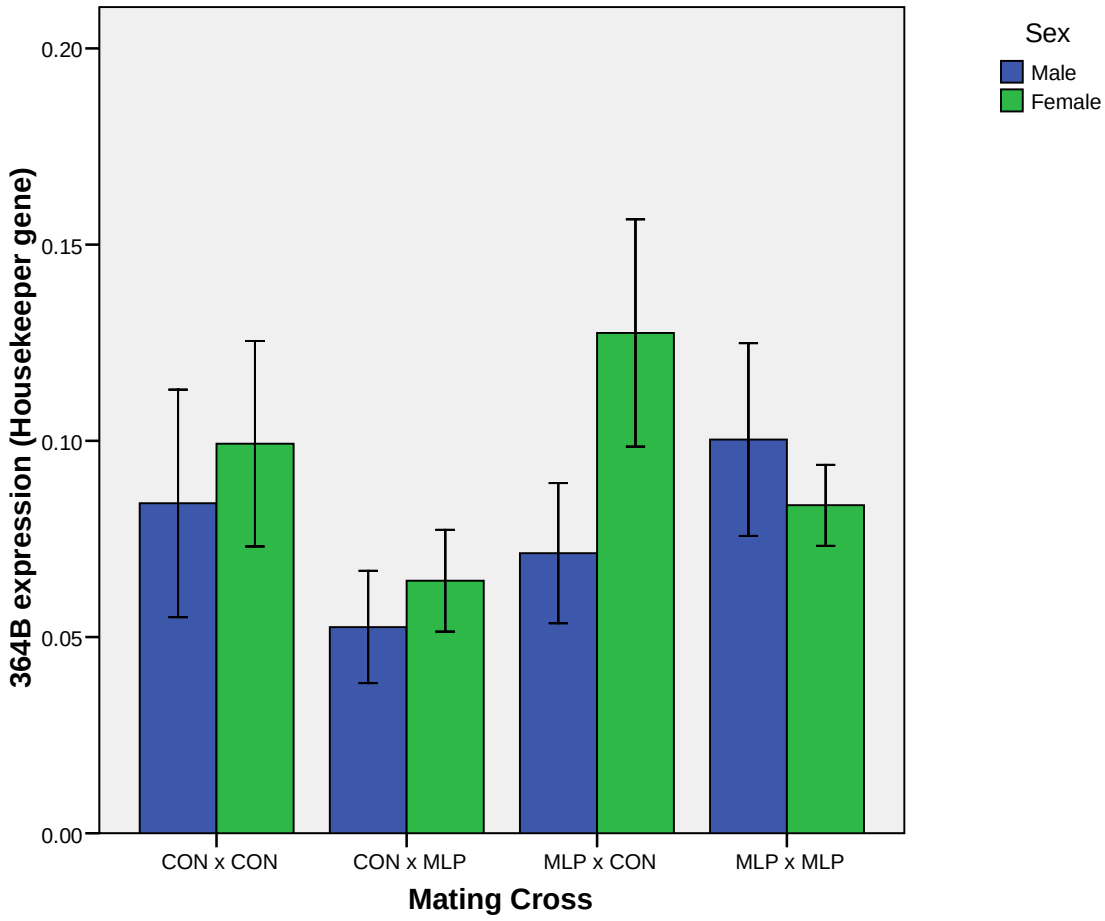


Figure 6.4 364B mRNA expression in 10 week old rat kidneys (F2). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

Figure 6.5 F3 generation 364B expression in 10 week old rat kidneys.

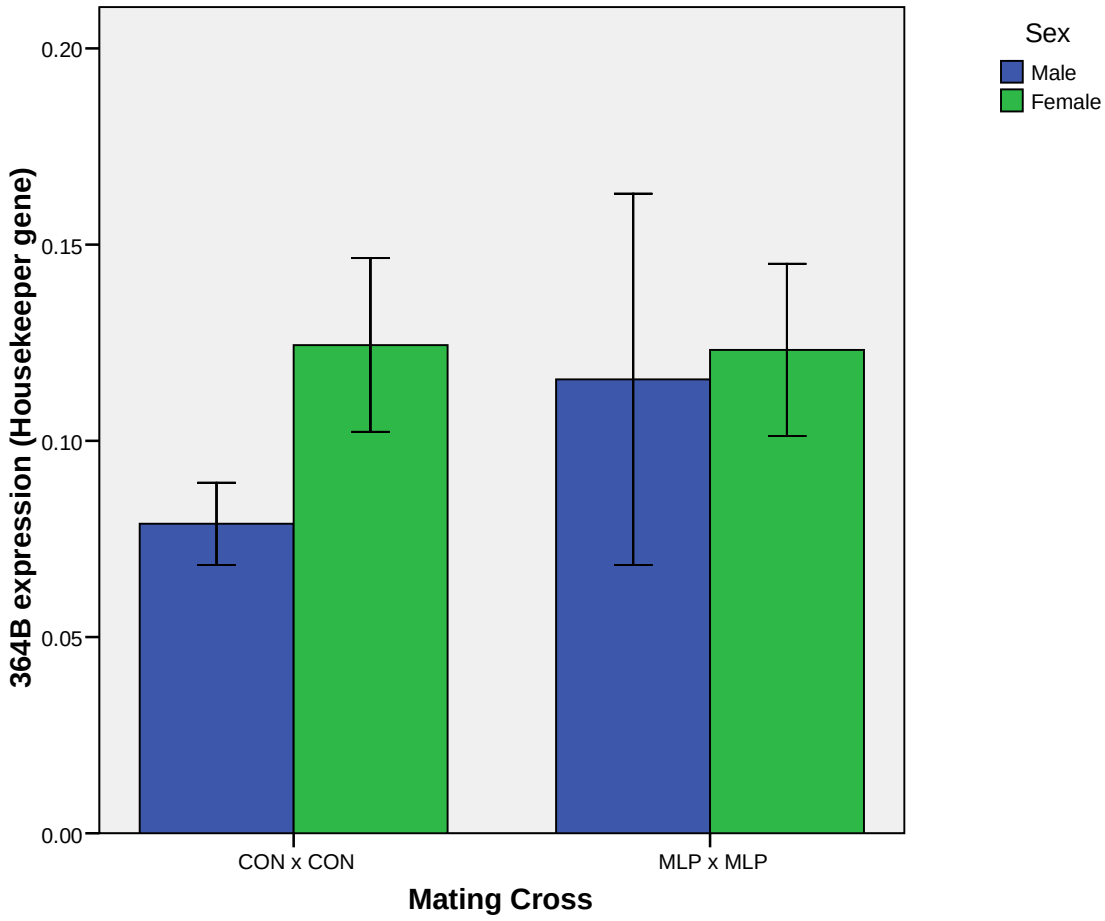


Figure 6.5 364B mRNA expression in 10 week old rat kidneys (F3). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

6.4.1.3.2 Matrix metallopeptidase 9 (MMP9) expression

MMP9 expression in 10 week old rat kidneys of the F1 generation (Figure 6.6) was measured relative to expression of 364B. After normalisation of the data, there was found to be no significant difference between control and MLP-exposed animals MMP9 mRNA expression at 10 weeks of age. Expression in male and female animals was also similar.

Figure 6.7 shows MMP9 mRNA expression in 10 week old rat kidneys of the F2 generation. After normalisation, MMP9 expression was similar in all mating crosses. Expression in male and female animals was also similar, however a significant interaction of sex and mating cross ($P<0.05$) was noted, with males from the MLP x MLP mating cross exhibiting lower MMP9 mRNA expression than any other mating cross.

F3 generation MMP9 mRNA expression (Figure 6.8) was significantly ($P<0.05$) lower in the MLP x MLP mating cross (approximately 50%) compared to controls. Expression in male and female animals was similar.

Figure 6.6 F1 generation MMP9 mRNA expression in 10 week old rat kidneys.

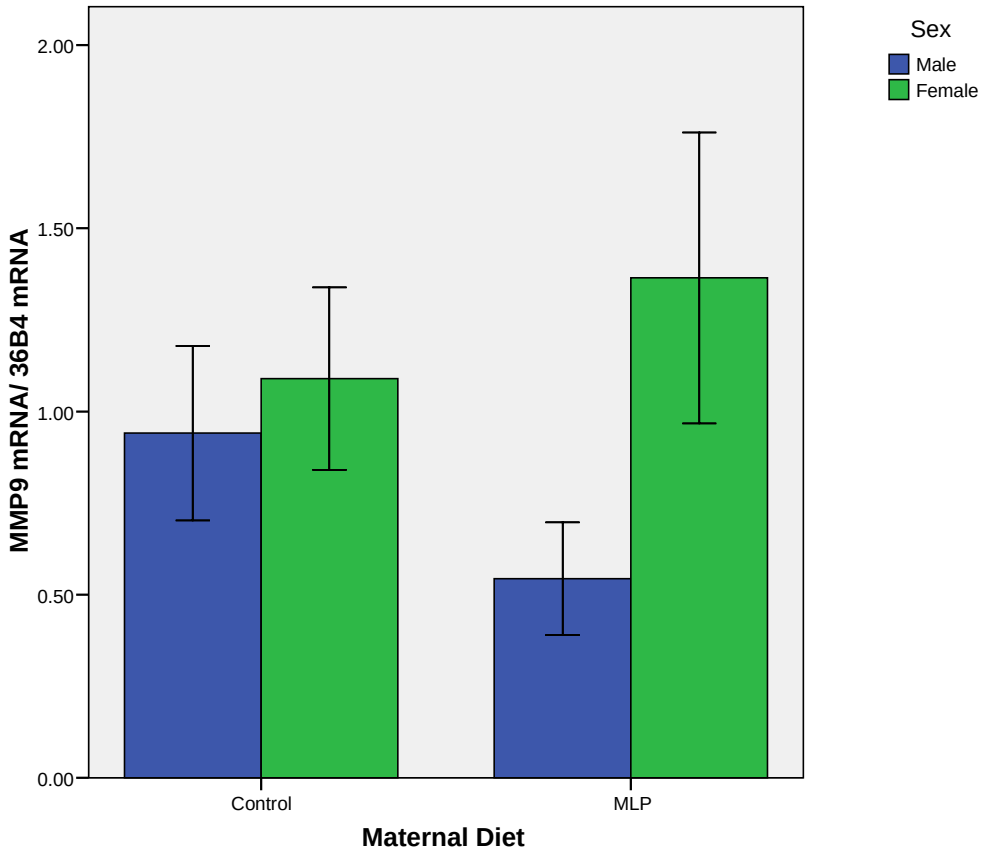


Figure 6.6 MMP9 mRNA expression in 10 week old rat kidneys (F1). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA.

Figure 6.7 F2 generation MMP9 mRNA expression in 10 week old rat kidneys.

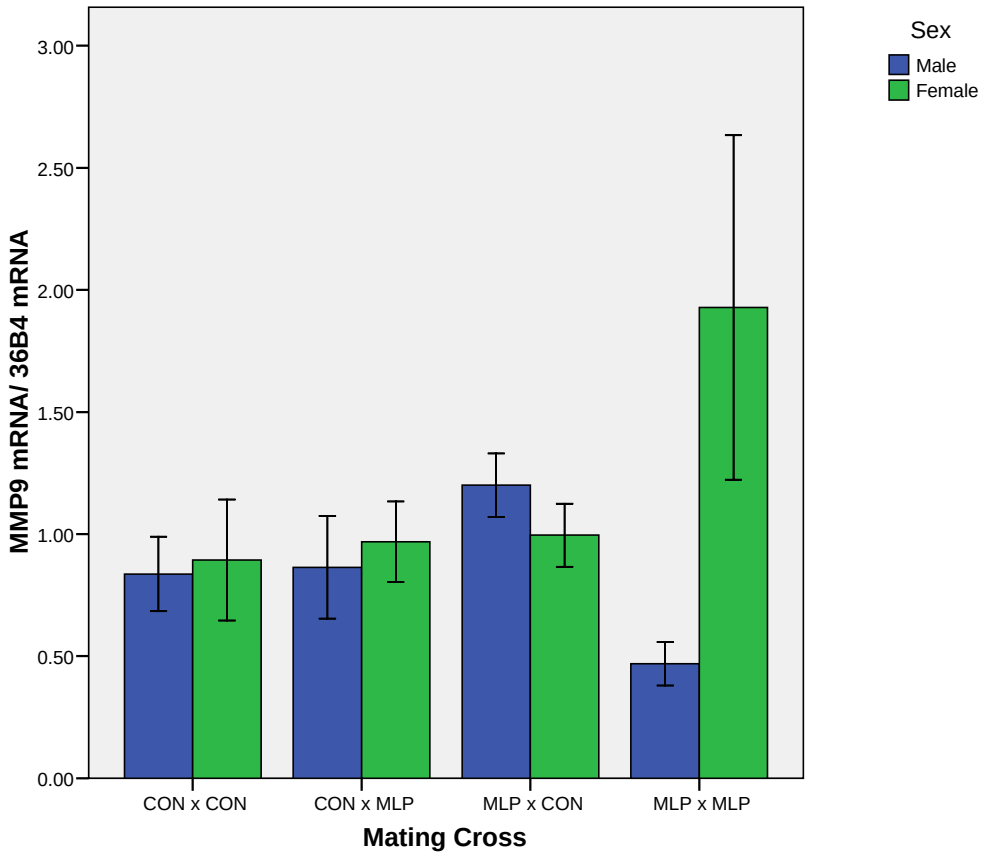


Figure 6.7 MMP9 mRNA expression in 10 week old rat kidneys (F2). Data is shown as mean ± SEM, n = 6 per group. A significant interaction of sex and mating cross ($P<0.05$) was noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

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Figure 6.8 F3 generation MMP9 mRNA expression in 10 week old rat kidneys.

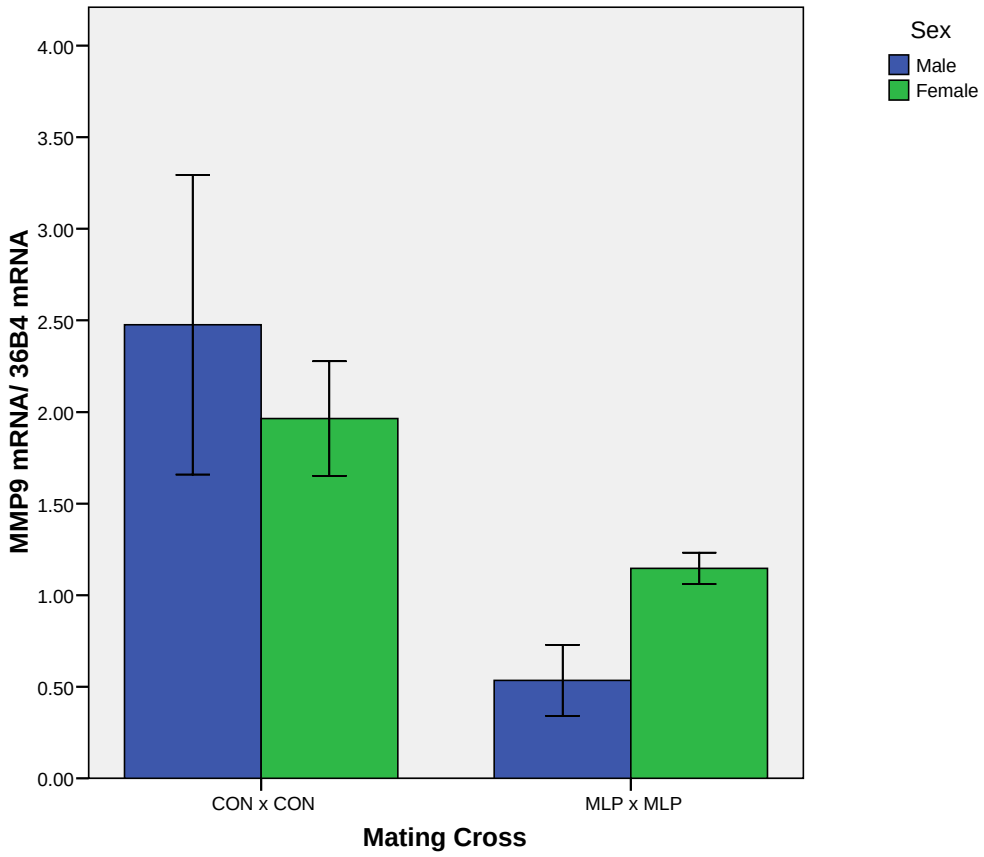

Figure 6.8 MMP9 mRNA expression in 10 week old rat kidneys (F3). Data is shown as mean ± SEM, n = 6 per group. A significant effect of mating cross (P<0.05) was noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

6.4.1.3.3 Granzyme B (GZMB) expression

GZMB mRNA expression in 10 week old rat kidneys of the F1 generation (Figure 6.9) was measured relative to the expression of 364B. After normalisation of the data, there was found to be a significant effect of maternal diet *in utero* upon GZMB expression. In contrast to the microarray results, MLP-exposed offspring had approximately 30% lower (P<0.05) GZMB expression compared to their control counterparts. Expression was similar among male and female animals.

In the F2 generation, GZMB expression (Figure 6.10) was similar irrespective of mating cross and sex. Figure 6.11 shows F3 generation GZMB mRNA expression in 10 week rat kidneys. After normalisation the data showed that expression values between mating crosses were similar. Female animals however, exhibited approximately 40-60% lower GZMB mRNA expression than male animals.

Figure 6.9 F1 generation GZMB mRNA expression in 10 week old rat kidneys.

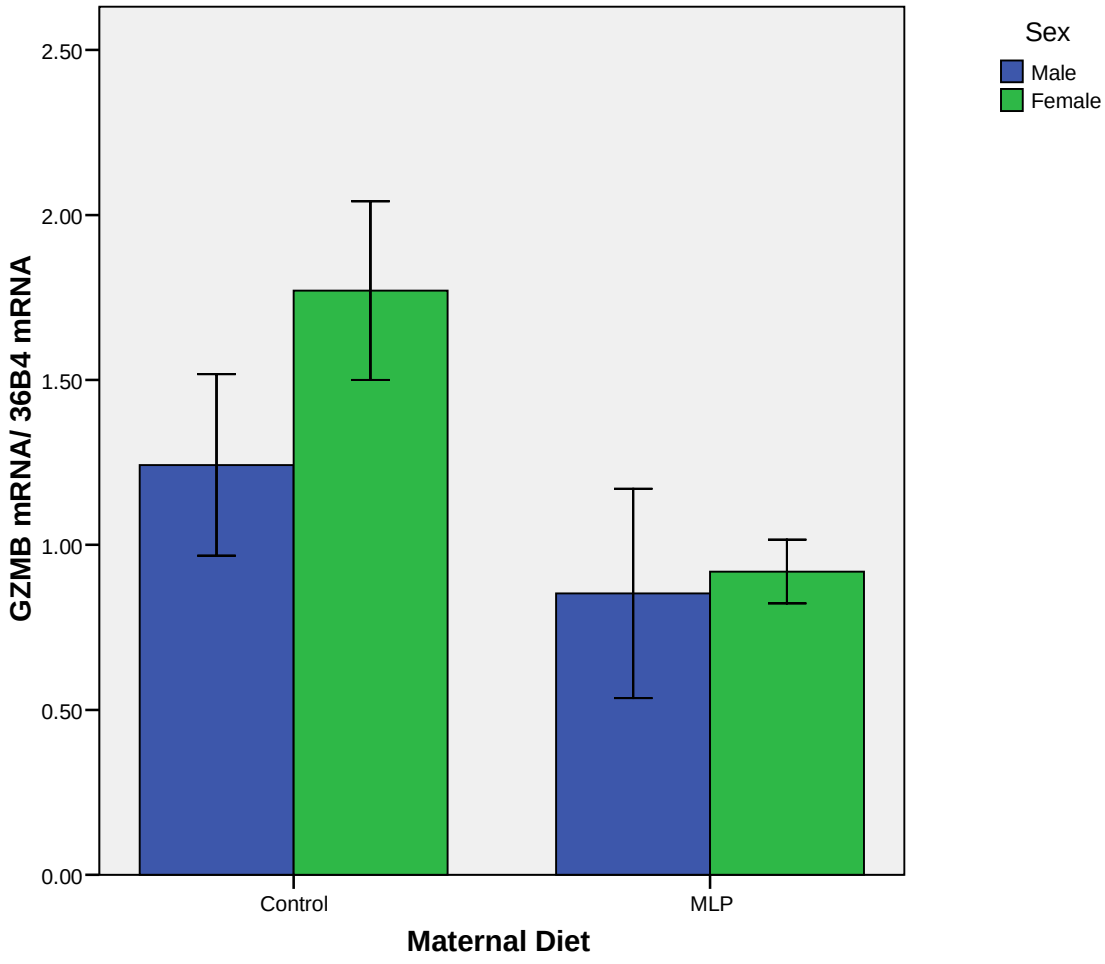

Figure 6.9 GZMB mRNA expression in 10 week old rat kidneys (F1). Data is shown as mean ± SEM, n = 6 per group. A significant effect of maternal diet ($P<0.05$) was noted. Data was analysed using a mixed model ANOVA.

Figure 6.10 F2 generation GZMB mRNA expression in 10 week old rat kidneys.

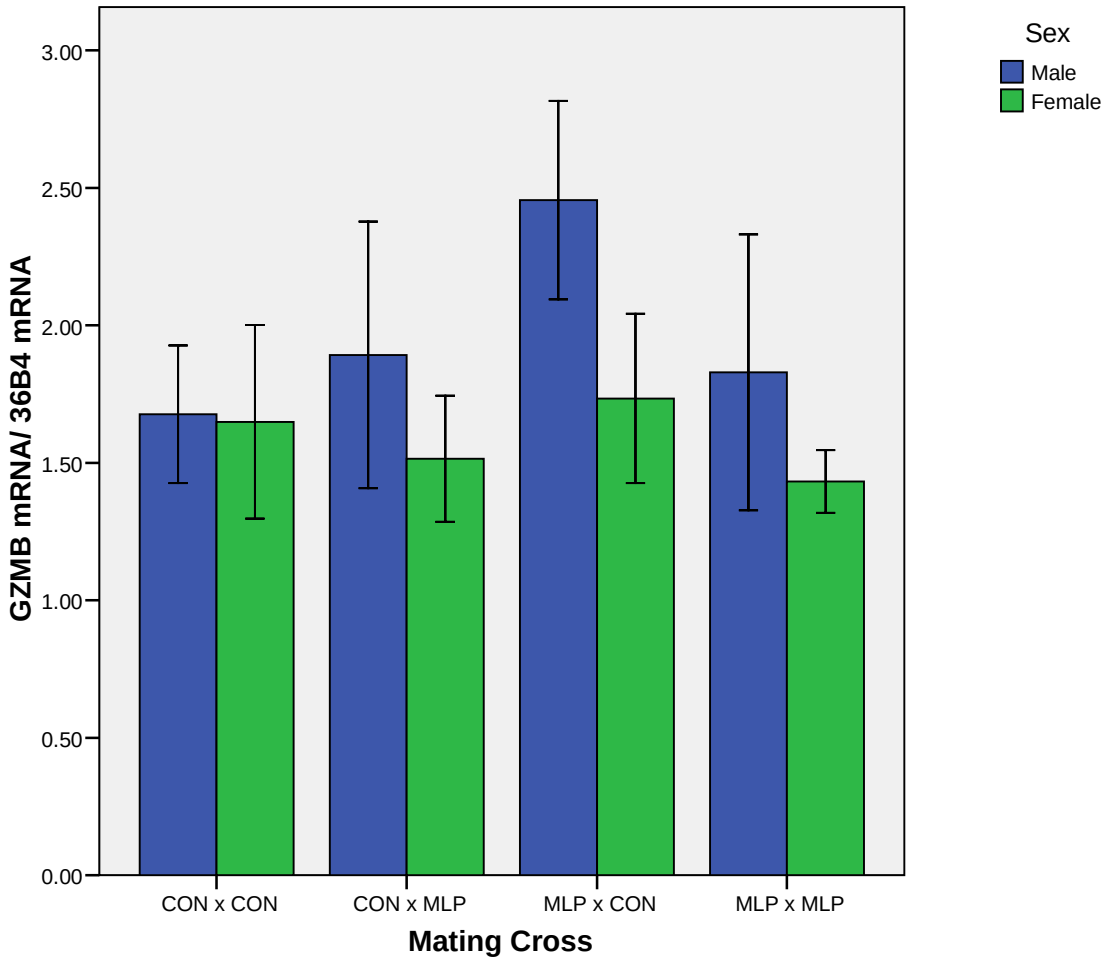

Figure 6.10 GZMB mRNA expression in 10 week old rat kidneys (F2). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

Figure 6.11 F3 generation GZMB mRNA expression in 10 week old rat kidneys.

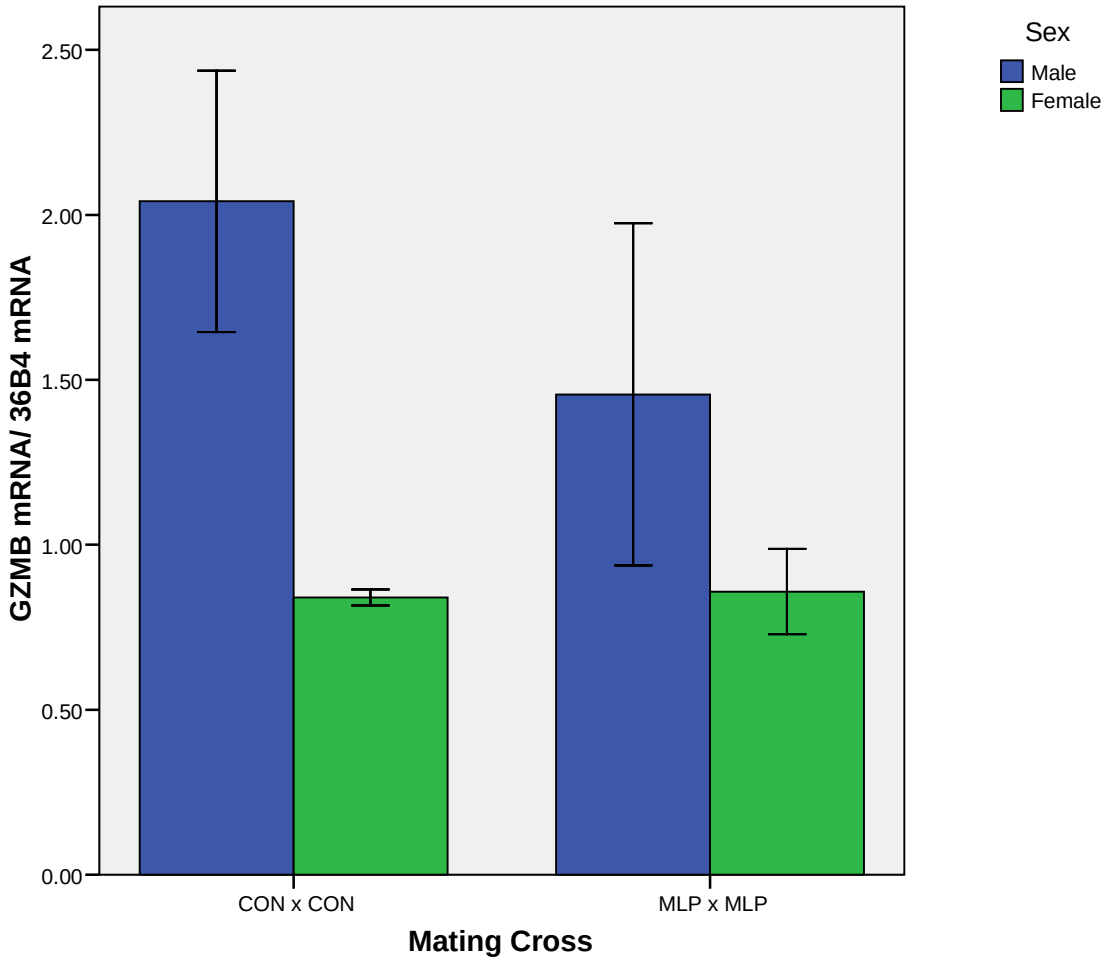

Figure 6.11 GZMB mRNA expression in 10 week old rat kidneys (F3). Data is shown as mean ± SEM, n = 6 per group. A significant effect of sex ($P<0.05$) was noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

6.4.1.3.4 Endothelial cell growth factor 1 (ECGF1) expression

F1 generation ECGF1 mRNA expression at 10 weeks of age (Figure 6.12) was measured relative to the expression of 364B. After normalisation the data showed no significant effects of exposure to a MLP diet *in utero.* Expression of mRNA in 10 week rat kidneys from male and female animals was similar.

As in the F1 generation, F2 generation ECGF1 expression at 10 weeks of age (Figure 6.13), was similar in all mating crosses irrespective of the sex of the animal.

There was also no effect of mating cross upon ECGF1 mRNA expression in the F3 generation (Figure 6.14). Expression levels in male and female animals were similar.

Figure 6.12 F1 generation ECGF1 mRNA expression in 10 week old rat kidneys.

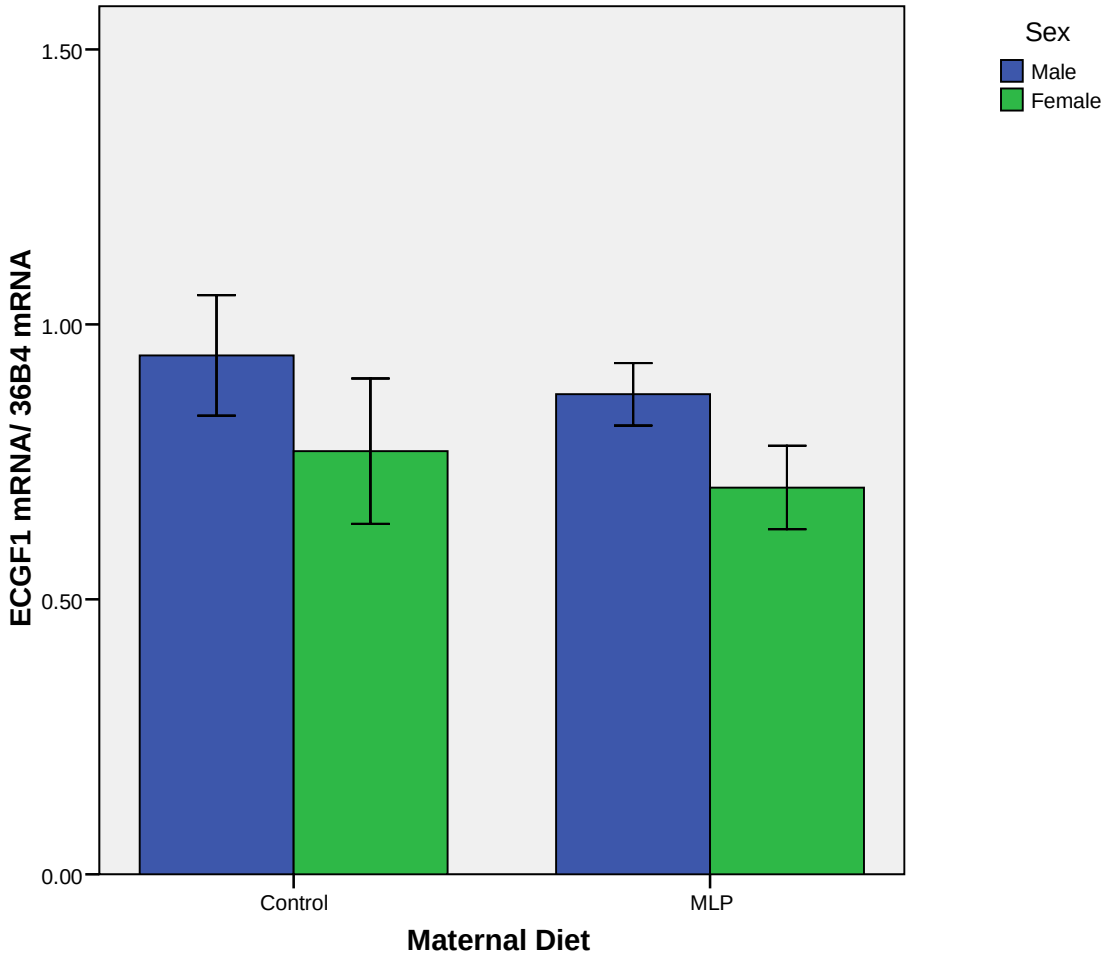

Figure 6.12 ECGF1 mRNA expression in 10 week old rat kidneys (F1). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA.

Figure 6.13 F2 generation ECGF1 mRNA expression in 10 week old rat kidneys.

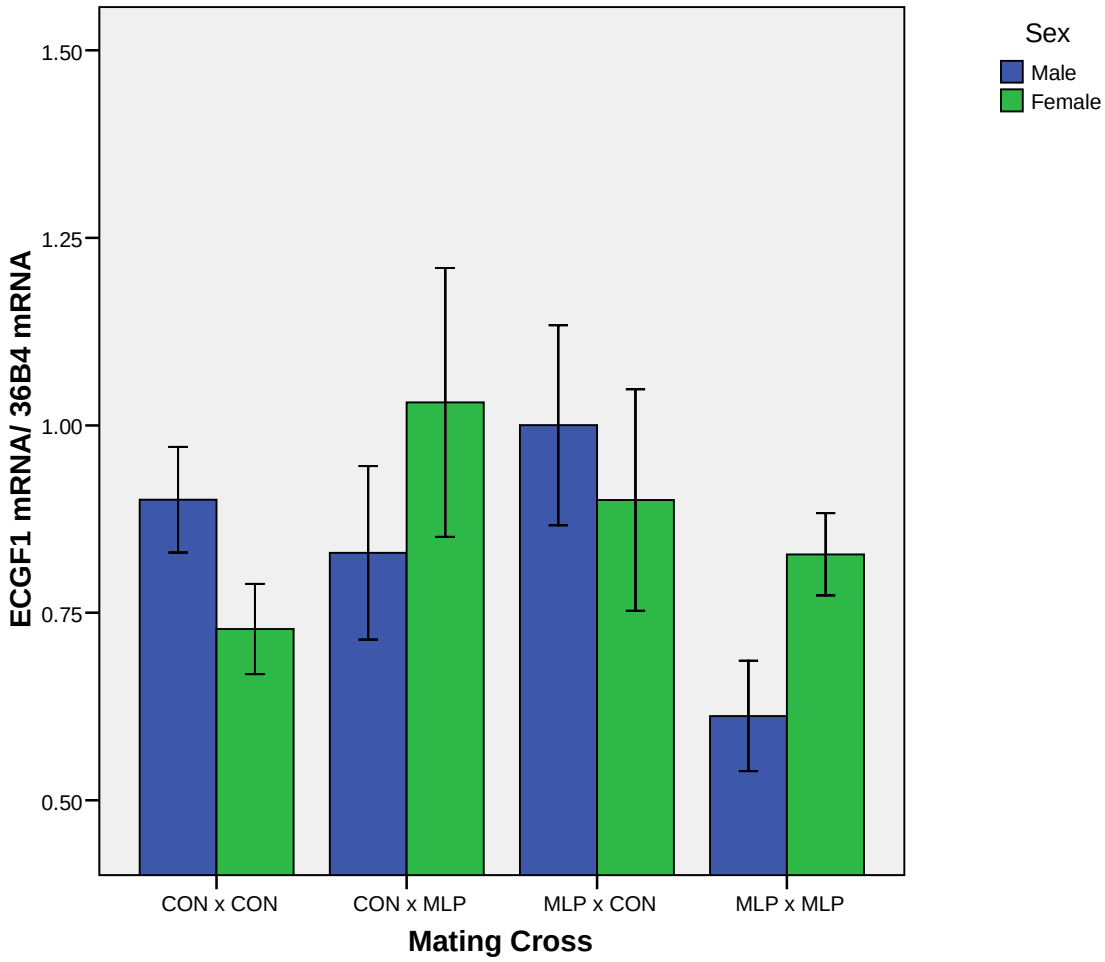

Figure 6.13 ECGF1 mRNA expression in 10 week old rat kidneys (F2). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

Figure 6.14 F3 generation ECGF1 mRNA expression in 10 week old rat kidneys.

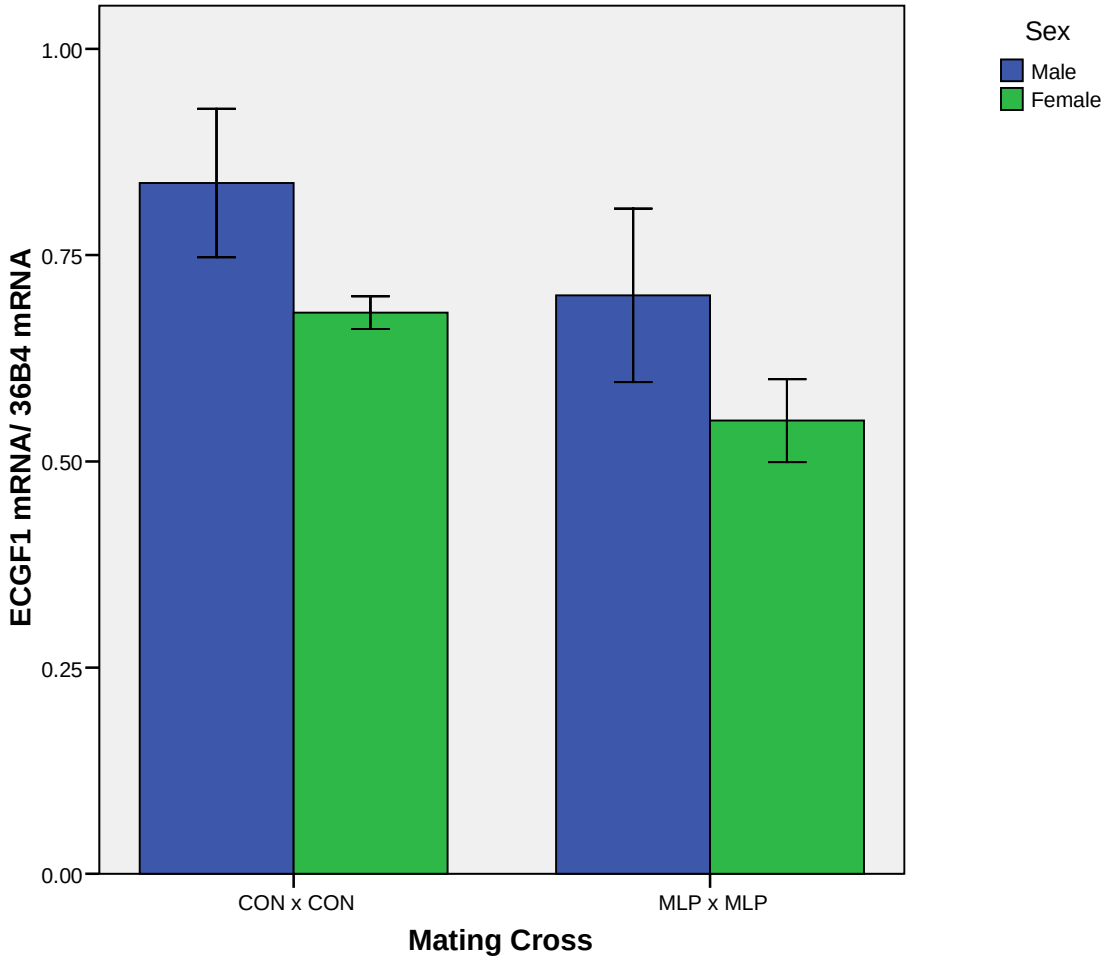

Figure 6.14 ECGF1 mRNA expression in 10 week old rat kidneys (F3). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

6.4.1.3.5 <u>Endothelin receptor type A (EDNRA) expression</u>

EDNRA expression in 10 week old rat kidneys of the F1 generation (Figure 6.15) was measured relative to expression of 364B. After normalisation and analysis of the data, there was found to be no significant difference in expression between control and MLP-exposed animals *in utero.* Expression levels between sexes however did differ ($P<0.05$). Male animals exhibited higher expression than female animals (approximately 20%) irrespective of maternal diet.

Figure 6.16 shows the expression of EDNRA mRNA in the kidneys of F2 generation 10 week old animals. Similar to the F1 generataion there was no effect of maternal diet/ mating cross upon EDNRA expression. Expression in male and female animals was similar. Expression of EDNRA in the F3 generation (Figure 6.17) was unaffected by either mating cross or sex of the animal.

Figure 6.15 F1 generation EDNRA mRNA expression in 10 week old rat kidneys.

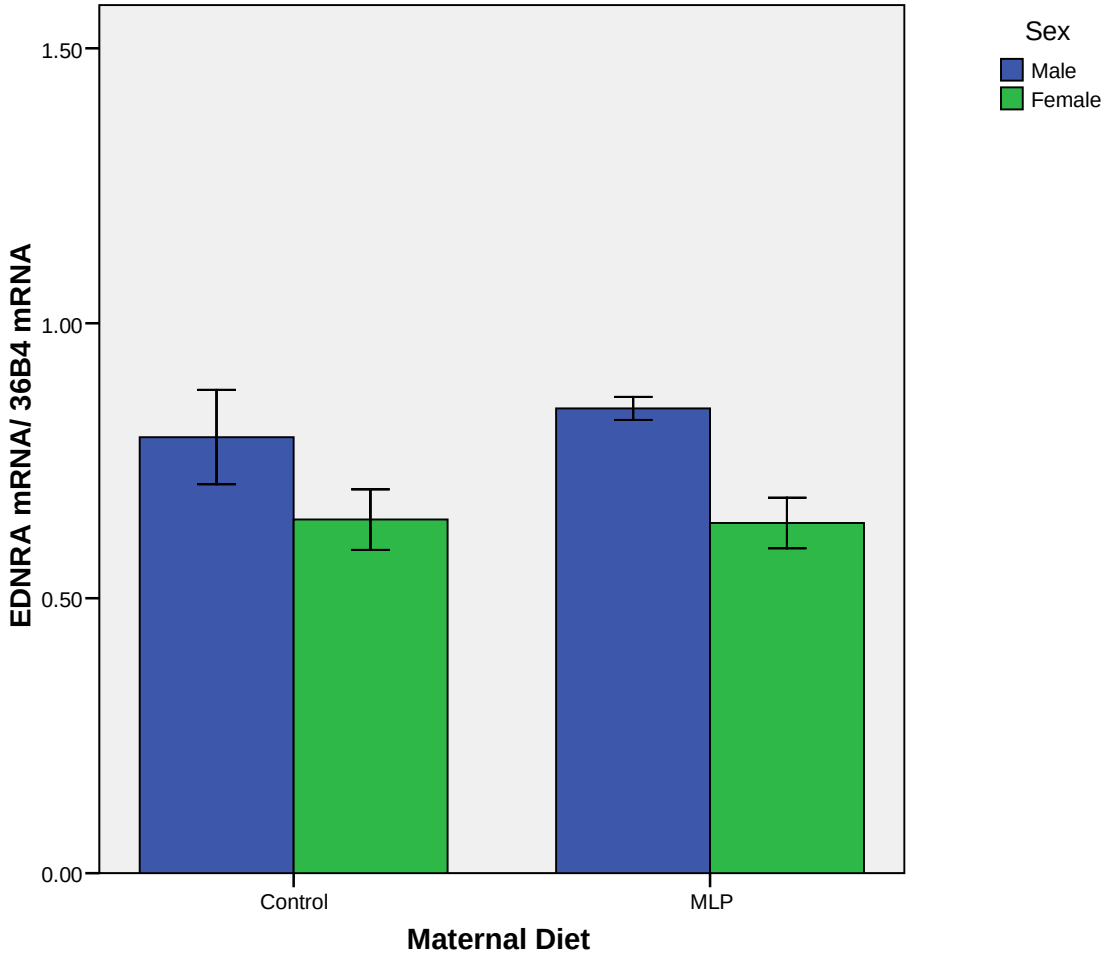

Figure 6.15 EDNRA mRNA expression in 10 week old rat kidneys (F1). Data is shown as mean ± SEM, n = 6 per group. A significant effect of sex ($P<0.05$) was noted. Data was analysed using a mixed model ANOVA.

Figure 6.16 F2 generation EDNRA mRNA expression in 10 week old rat kidneys.

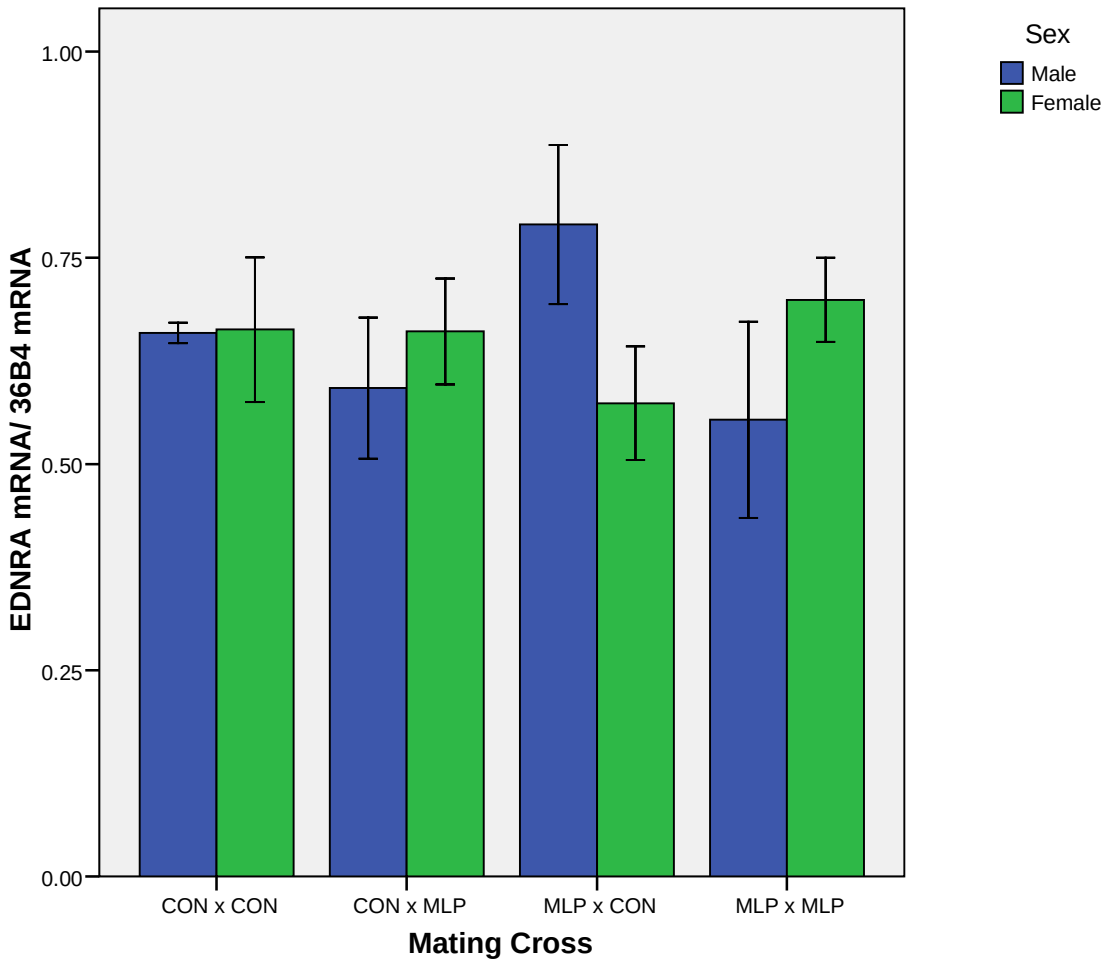

Figure 6.16 EDNRA mRNA expression in 10 week old rat kidneys (F2). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

Figure 6.17 F3 generation EDNRA mRNA expression in 10 week old rat kidneys.

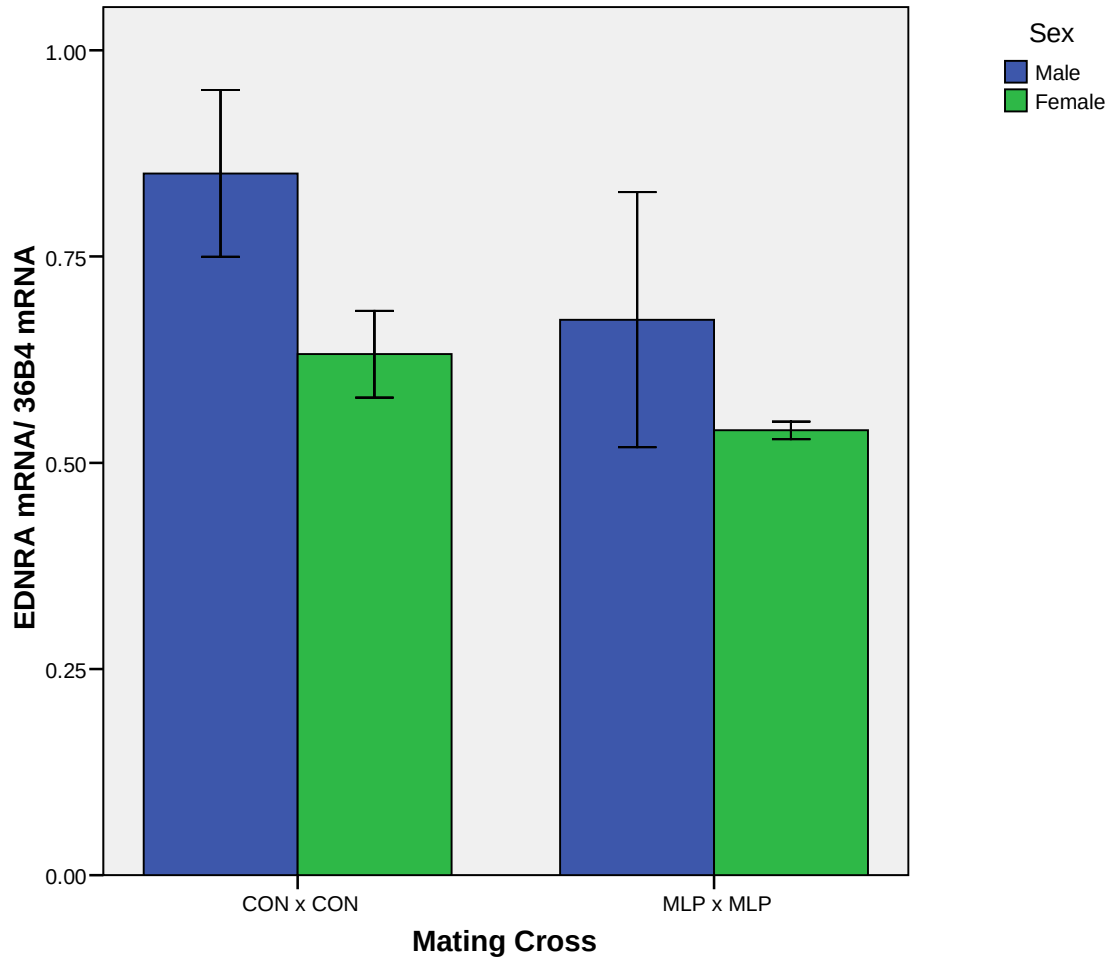

Figure 6.17 EDNRA mRNA expression in 10 week old rat kidneys (F3). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

6.4.1.3.6 Insulin-like growth factor 2 (IGF-2) expression

Figure 6.18 shows IGF-2 expression in 10 week old rat kidneys of the F1 generation relative to expression of 364B. After normalisation and analysis of the data, it was apparent that MLP-exposed offspring exhibited lower IGF-2 mRNA expression ($P<0.05$) than control animals (approximately 30- 50%). Expression levels in male and female animals were similar.

IGF-2 mRNA expression in 10 week old rat kidneys from the F2 generation (Figure 6.19) was unaffected by both mating cross and sex of the animals. Similarly in the F3 generation IGF-2 mRNA expression levels in 10 week old rat kidneys (Figure 6.20) were unaffected by both mating cross and sex.

Figure 6.18 F1 generation IGF-2 mRNA expression in 10 week old rat kidneys.

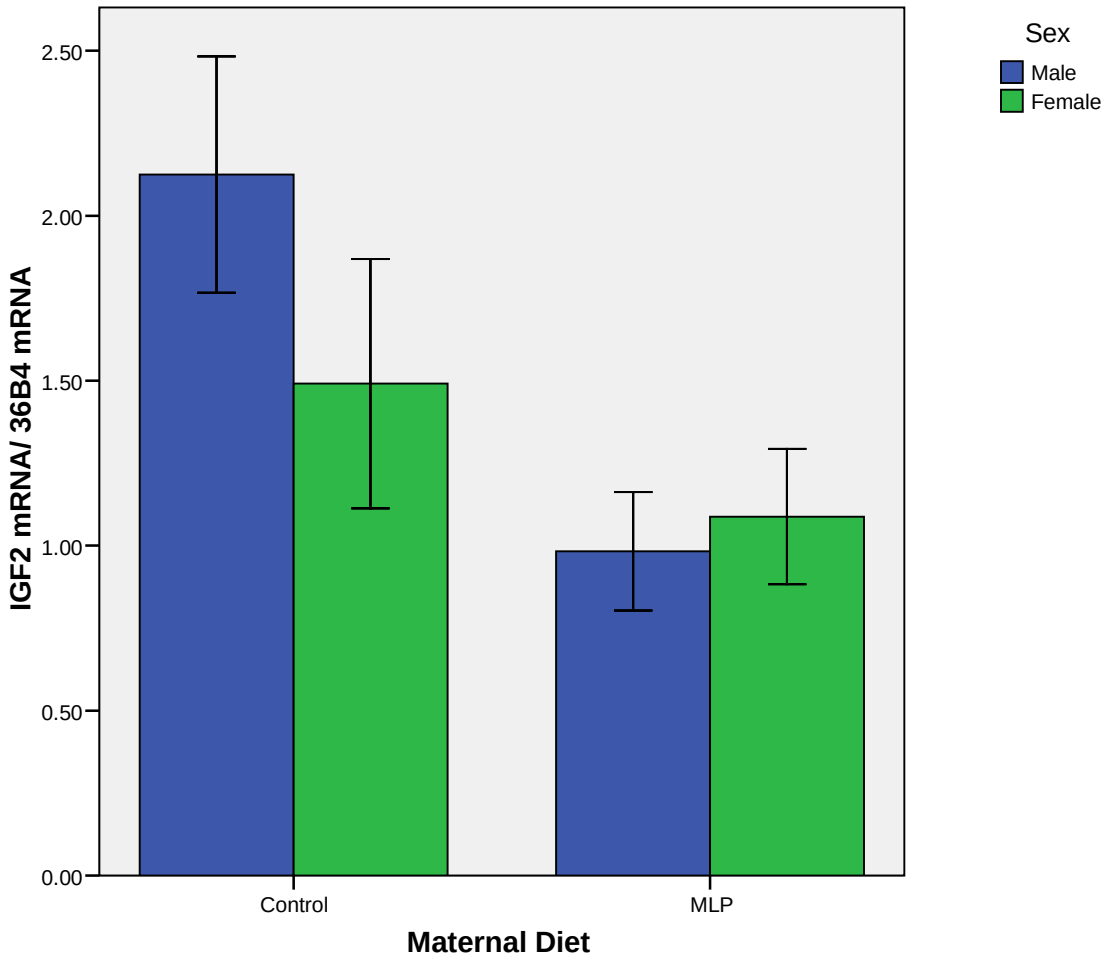

Figure 6.18 IGF-2 mRNA expression in 10 week old rat kidneys (F1). Data is shown as mean ± SEM, n = 6 per group. A significant effect of maternal diet ($P<0.05$) was noted. Data was analysed using a mixed model ANOVA.

Figure 6.19 F2 generation IGF-2 mRNA expression in 10 week old rat kidneys.

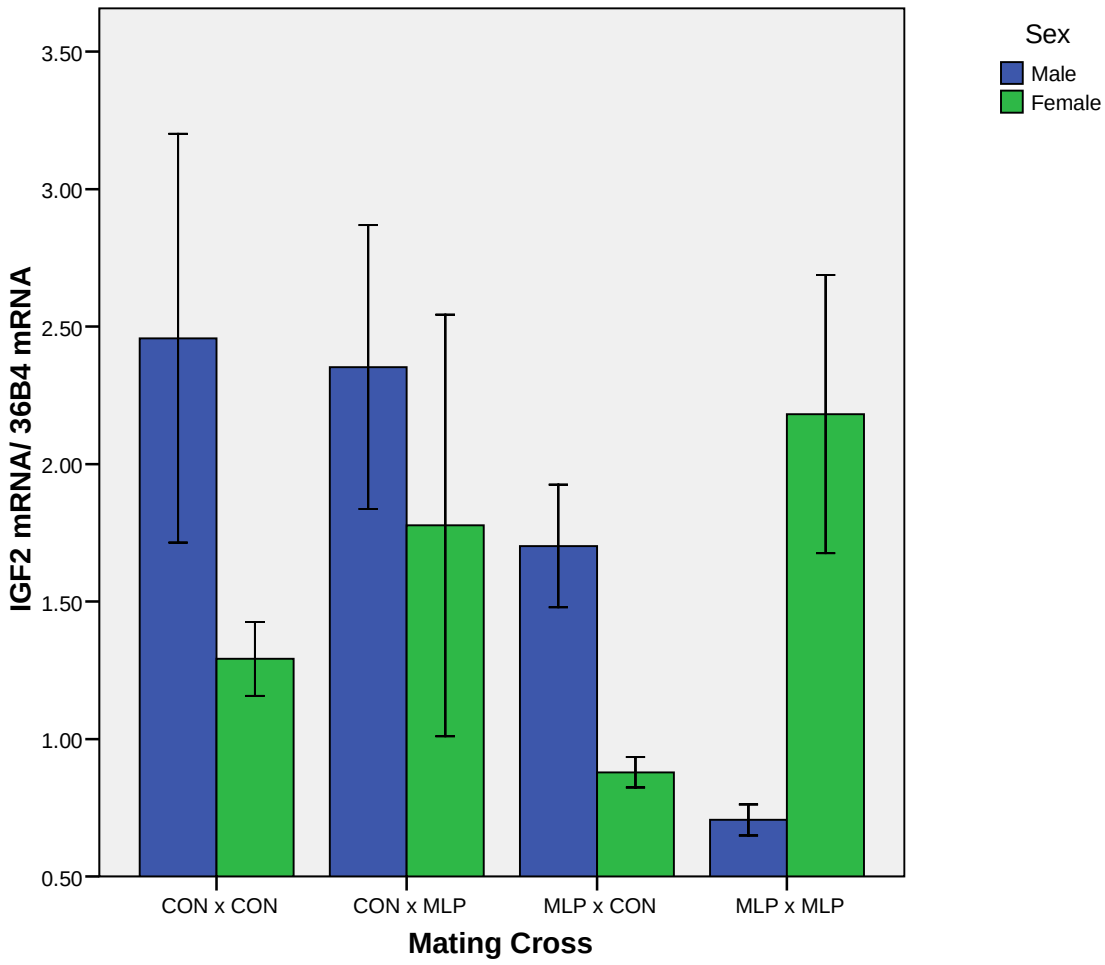

Figure 6.19, IGF-2 mRNA expression in 10 week old rat kidneys (F2). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

Figure 6.20 F3 generation IGF-2 mRNA expression in 10 week old rat kidneys.

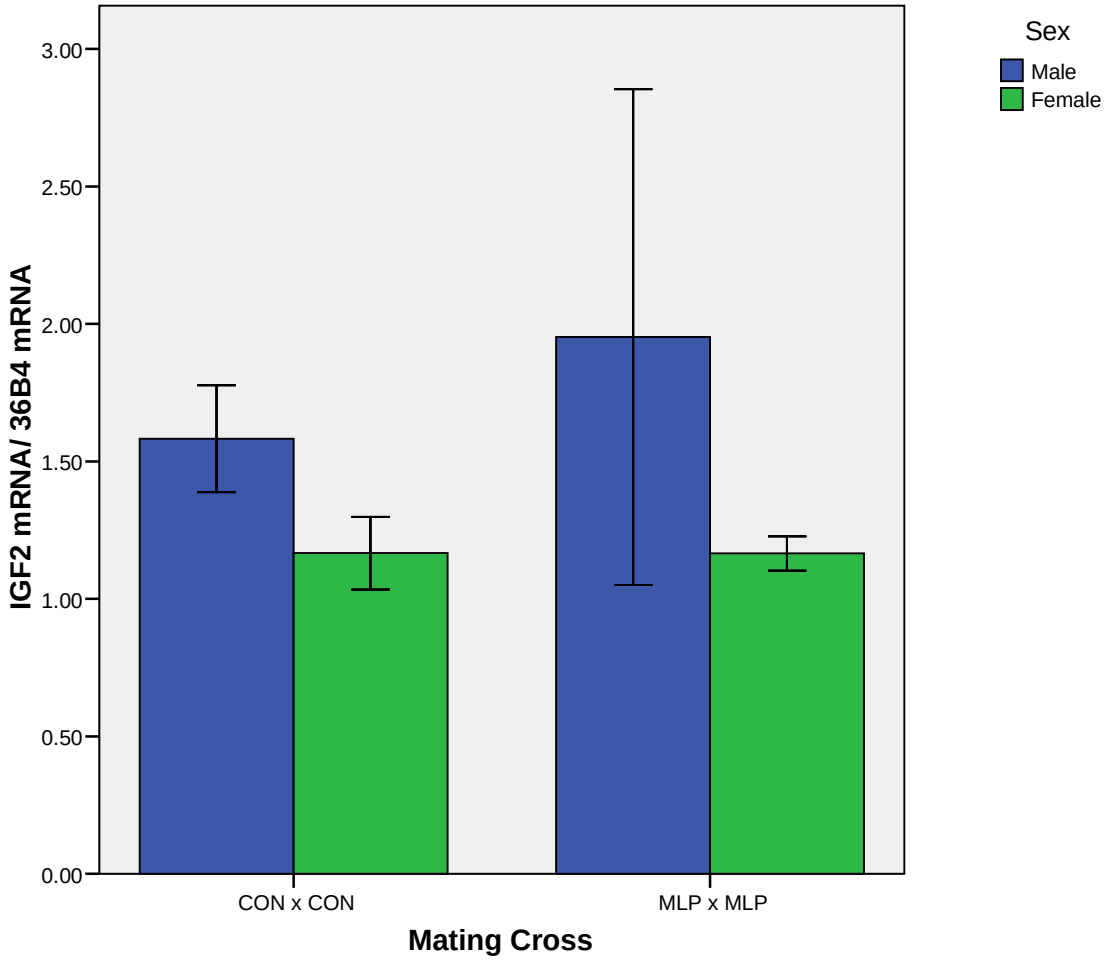

Figure 6.20, IGF-2 mRNA expression in 10 week old rat kidneys (F3). Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using a mixed model ANOVA. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*.

6.4.2 DNA Methylation

Global DNA methylation was assessed using the cytosine extension assay detailed in section 2.7.2. Figure 6.21 shows the methylation results as a ratio of *Hpa*II to *Msp*I, which is a measure of total percentage methylation.. There was no effect of maternal diet/ mating cross on DNA methylation in any generation. The effects of sex and postnatal HF feeding were not analysed due to time constraints.

Figure 6.21 DNA methylation in 10 week old rat kidneys.

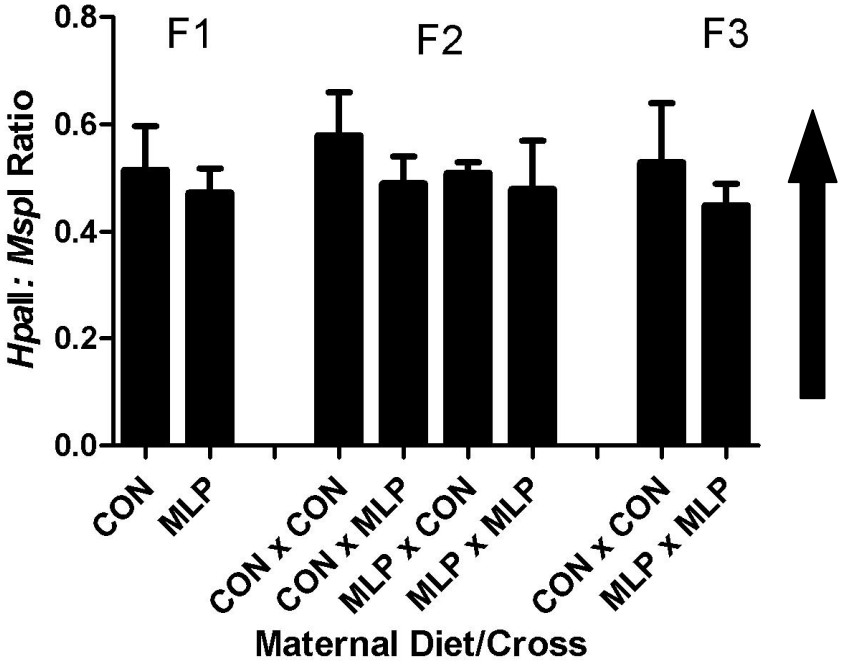

Figure 6.21 DNA methylation status of 10 week old rat kidneys. Data is shown as mean ± SEM, n = 6 per group. No significant effects were noted. Data was analysed using Univariate analysis. For F2 and F3 crosses the dietary origin of the male parent is shown before the female parent, e.g. Con x MLP indicates a cross between a male exposed to control diet and a female exposed to a MLP diet *in utero*. Arrow indicates increasing methylation.

6.5 Discussion

6.5.1 Summary of findings

Current thinking is that disease arises due to a mismatch between the prenatal and postnatal environments (Gluckman and Hanson, 2004). Evidence demonstrates that rapid catch-up growth following prenatal growth restriction is a predictor for the metabolic syndrome (Eriksson *et al.*, 2003). It has been argued that deficits in maternal nutrition during pregnancy are ultimately transmitted to the grandchildren (James, 2002). This is likely to involve the alteration of DNA through either DNA methylation and/ or histone modification (Isles, 2005; Waterland *et al.*, 2007). Work within this thesis has demonstrated that MLP exposure during gestation has implications for the health of future generations. The current chapter aims to identify possible gene targets involved in the production of the phenotype associated with the low protein diet.

Table 6.4 Summary of findings

	Generation		
	F1	**F2**	**F3**
qRT-PCR (MMP9)	No difference in expression	Decreased expression in MLP male animals	Decreased expression in MLP x MLP mating cross, no effect of sex
qRT-PCR (GZMB)	Decreased expression in MLP maternal diet group, no effect of sex	No change with mating cross or sex	No effect of mating cross, expression decreased in females
qRT-PCR (ECGF1)	No change with maternal diet or sex	No change with mating cross or sex	No change with mating cross or sex
qRT-PCR (EDNRA)	No effect of maternal diet, increased expression in males	No change with mating cross or sex	No change with mating cross or sex
qRT-PCR (IGF-2)	Decreased expression in MLP groups, no effect of sex	No change with mating cross or sex	No change with mating cross or sex
DNA methylation	No change with maternal diet	No change with mating cross	No change with mating cross

Table 6.4 shows a brief summary of the findings from the assessment of gene expression and DNA methylation analysis in the F1, F2 and F3 generations

6.5.2 Effect of prenatal protein restriction on gene expression

Maternal restriction of protein has previously been shown to induce hypertension from 4 weeks of age (Langley-Evans *et al.*, 1994) coupled with a reduction in nephron complement (Langley-Evans *et al.*, 1999a) in the F1 generation. Although the development of hypertension is clearly multifactorial, hypertension is intrinsically linked to kidney function (Gilbert *et al.*, 2005; Langley-Evans, 2006; Langley-Evans *et al.*, 1999a; Mackenzie, 1995; Mackenzie *et al.*, 1996). Previous chapters within this thesis have demonstrated that a hypertensive phenotype is transmitted from the F1 to the F2 generation. To date there has been very little data explaining the mechanisms behind these programmed changes. The original aim of this chapter was to discover and analyse potential gene targets involved in the reduced nephron complement and hypertensive phenotype outlined within this thesis. This study is unique in that it is the first to assess the effect of *in utero* exposure to MLP diet on renal gene expression in 3 successive generations.

In order to select possible gene targets a decision was made to employ the use of microarray technology to highlight genes that were differentially expressed between groups within each generation of animals. From these studies we hoped to identify genes in which mRNA expression was significantly changed in the F1 and F2 generations, where we see the hypertensive phenotype and possibly continuing into the F3 generation. Microarray findings would be backed up by qRT-PCR studies. To begin this process the pathway focussed Oligo GEArray DNA Microarray for rat endothelial cell biology was selected to analyse the expression of 113 genes associated with permissibility and vessel tone, angiogenesis, endothelial cell activation and endothelial cell injury. A pathway focussed DNA microarray was chosen over a whole genome array as arrays which target the whole genome often

provide a wealth of data, which can be difficult to interpret. Results from more focussed microarrays often provide insights into what is happening with in a specific panel or family of genes.

Results (Tables 6.1-6.3) from the microarray analyses highlighted 4 potential gene targets that had higher than a 2 fold increase or decrease between groups, which were common between the F1 and F2 generations, and 1 gene that was down-regulated 50% in all 3 generations These were Matrix metallopeptidase 9 (MMP9), Granzyme B (GZMB), Endothelial cell growth factor 1 (ECGF1), Endothelin receptor type A (EDNRA) and Interlukin 7 (Il7). As previously stated, we were unable to analyse the expression of Il7 by qRT-PCR. Analysis of the remaining genes identified by the microarray by qRT-PCR showed no significant effect of maternal diet on the expression of MMP9, ECGF1 and EDNRA in the F1 generation. However, post hoc power calculations revealed that MMP9 and ECGF1 analyses were underpowered. Although sample means in the Con and MLP groups were substantially different (15%), standard deviations were relatively large and an increased sample size would have been needed in order for the difference to be statistically significant (Table 6.5). In contrast, the difference between means for EDNRA was very small and the risk of Type II error in this data set is low. GZMB expression was significantly decreased in the F1 generation, but this effect was in the opposite direction to the results observed in the microarray.

Similarly there was no effect of mating cross on the expression of any of the selected genes within the F2 generation, with the exception of MMP9 in which mRNA expression levels were lower in males from the MLP x MLP mating cross. Again, post hoc analysis of power revealed that the analysis of ECGF1 was underpowered (Table 6.5).

The F3 generation exhibited a reduced MMP9 expression in the MLP x MLP mating cross. The expression of all the other genes were unaffected by dietary factors. Post hoc analysis suggested that the analysis of the expression of GZMB and EDNRA were underpowered (Table 6.5).

Table 6.5 Summary of data for power calculations.

Gene	Mean group 1	SD group 1	Mean group 2	SD Group 2	Samples required for statistical significance
F1					
MMP9	1.189	1.056	1.015	0.575	**409**
ECGF1	1.017	0.895	0.857	0.297	**295**
EDNRA	0.697	0.240	0.718	0.185	**1735**
F2					
GZMB	1.663	0.714	1.699	1.141	**11484**
ECGF1	1.070	0.775	0.802	0.372	**88**
EDNRA	0.733	0.297	0.729	0.438	**158700**
F3					
GZMB	1.566	0.912	1.379	1.246	**586**
ECGF1	0.769	0.198	0.810	0.393	**1008**
EDNRA	0.712	0.220	0.528	0.298	**34**

Table 6.5 SD = standard deviation. F1 generation group1 = MLP, group 2 = Con. F2 and F3 generations group 1 = MLP x MLP, group 2 = Con x Con. The probability of a type I error (α) was set at 0.05 and of a type II error (β) set at 0.10, to give a power (β-1) of 0.90. Means and Standard deviations from this table were used to calculate the number of samples required for statistical significance.

The involvement of the expression of GZMB, ECGF1 and EDNRA in the development of the MLP phenotype observed within this study can therefore be ruled out, as none of the genes showed altered expression in both the F1 and F2 generations. The involvement of MMP9 can also largely be disregarded as, although there was lower expression in both the F2 generation MLP x MLP males and the F3 generation MLP x MLP mating cross animals, the hypertensive phenotype observed

within this thesis is present in both the male and female animals. The qRT-PCR results of MMP9 were especially unexpected as previous research has indicated that MMP9 is produced at the first stage of kidney embryogenesis *in vivo* and it is also required for branching morphogenesis *in vitro* (Lelongt *et al.*, 1997; Pohl *et al.*, 2000). Further to this, MMP9 deficient mice exhibit a 12% reduction in nephron number and at 12 months of age develop renal impairment and atrophy (Lelongt and Ronco, 2002). These findings Indicate that MMP9 is required for nephron formation and preservation of renal function. One possible reason for the negative results for MMP9 is the age of the animals studied (10 weeks postnatal). Previous research has indicated that in a mouse model MMP9 expression could not be detected above background level in the kidney from prenatal day 12 onward (Reponen *et al.*, 1994). The timing of measurements is likely to be critical when examining gene expression. Events *in utero* have the potential to trigger a phenotype to develop. Further to this gene changes *in utero* may be transient (Thompson *et al.*, 2005) and therefore not observable once the phenotype has fully established.

A drawback of the work within this chapter is the poor agreement of the qRT-PCR studies with the microarray studies, for example Gzmb mRNA expression in the F1 generation was reduced when analysed by qRT-PCR, however when analysed by microarray mRNA expression was greater than in controls. These results reinforce that although microarray technology is a useful tool for selecting candidate genes, all analyses need to be backed up by qRT-PCR analyses to verify the results. Although the expression of none of the genes analysed within this investigation were found to be altered by exposure to MLP, it is important to note that there are several limitations associated with the use of a pathway focussed microarray technology utilised within this investigation. For example the array is, by definition, targeted and

therefore does not analyse the entire genome, and therefore there were limits to the analysis. There is also the possibility that we selected an inappropriate target array, an array focussed on the extracellular matrix or a genome wide array looking at thousands of genes may have yielded more targets for follow up. Although this technique often provides such a wealth of data it can be challenging to select candidate genes. In the current investigation we decided to utilise the pathway focussed endothelial cell microarray as we believed that as we had a hypertensive phenotype that also displayed a reduced nephron complement this array would be the best suited for identifying possible candidate genes. Further to the limitations of the microarray another potential drawback to the work within this chapter is that due to expense and time constraints we were unable to analyse protein expression to back-up the findings from both the microarray and qRT-PCR studies.

In addition to the investigation of genes highlighted by the microarray data, recent research has indicated that individuals exposed to the Dutch Hunger Winter of 1944-45 have differing expression of insulin-like growth factor 2 (IGF-2), compared to non-exposed controls (Heijmans *et al.*, 2008). Analysis of IGF-2 by qRT-PCR indicated that its expression was not linked to the phenotype observed within this investigation as although a lower expression was noted in MLP- exposed offspring of the F1 generation, no difference in expression was noted within the F2 generation. It is also worth noting that the lower expression noted within the current study is the opposite to the greater expression levels noted within the human study (Heijmans *et al.*, 2008). Although all the genes investigated within this study were found to be unaffected by MLP exposure it is important to note that the phenotype noted within this study must be preceded by modifications at the molecular level. Recent research by Abdel-Hakeem and colleagues (2008) has demonstrated several possible gene

targets involved in controlling transcription and growth in fetal nephrogenesis including glial cell line-derived neurotrophic factor (GDNF) which has a critical role in promoting ureteric bud (UB) branching (Abdel-Hakeem *et al.*, 2008). As previously stated a possible limitation of this study is the time at which the kidney samples were taken (10 weeks). Collection of kidney samples during the final stages of gestation (day 20) would have been advantageous as it would have allowed us to evaluate expression at a time during which UB branching and mesenchymal transformation were occurring. The analysis of the whole kidney within the current investigation may have overlooked regional effects, therefore, the study of gene expression within the different compartments of the kidney during the different stages of development would also have been advantageous.

The analysis of methylation indicated no significant differences between groups. The cytosine extension assay utilised within this investigation is a high throughput method for the analysis of global methylation. The results obtained were unexpected as the induction of an altered phenotype by prenatal undernutrition is thought to involve changes in the epigenetic regulation of specific genes (Goldberg *et al.*, 2007; Whitelaw, 2006; Whitelaw and Whitelaw, 2006). Previous research has demonstrated that protein restriction induces changes in hepatic PPARα promoter (Lillycrop *et al.*, 2008) which are subsequently passed onto a second generation (Burdge *et al.*, 2007a).

It is important to note that the cytosine extension assay has one specific limitation. The assay analyses global methylation. Therefore large changes in methylation status at specific promoters may go unnoticed as methylation of the genome as a whole is unchanged. The current study would therefore benefit from the analysis of methylation status from a gene-specific view-point, employing the use of

either genome wide methods of analysis such as Restriction Landmark Genome Scanning (RLGS), or candidate gene methods such as Direct bisulfite Sequencing. Although after analysis, results from cytosine extension assay proved not to be significant, there was however a trend of hypomethylation in all 3 generations. This is consistent with the work of Sinclair and colleagues (2007) who analysed over 1400 CpG sites and noted a 4% (57 loci) change in total methylation status, 88% of which were hypomethylated (Sinclair *et al.*, 2007). Interestingly Maloney and colleagues (2007) who also utilized the cytosine extension assay in order to analyse DNA methylation, after feeding rats diets deficient in folate, methionine and choline during pregnancy found no significant change in methylation (Maloney *et al.*, 2007). It is possible that the methylation analysis within the current investigation was underpowered and if numbers were increased a significant value might be obtained.

Work within this investigation has demonstrated the passage of a phenotype consisting of high blood pressure and reduced nephron complement from F1 generation to the F2 generation via both the maternal and paternal lines. Although there was no change in the expression of genes at the time points monitored in this investigation, on reflection some of the RT-PCR studies were underpowered which may explain some of the negative results. However, it must be noted that the dysregulation of renal genes involved in endothelial cell function and UB branching at differing stages of development are likely to be involved in providing new insights into our understanding the molecular machinery behind the phenotype observed within this thesis.

7.0 General discussion and future prospects

The original fetal origins hypothesis conceived by Barker and colleagues (1989) has both evolved and expanded over the last few decades (Barker, 1992; Barker, 1994; Hales, 2001) and now encompasses an abundance of data from both epidemiological studies (Ravelli, 1998, 1999; Roseboom, 2000, 2001) and animal models (Kind, 1999; Langley-Evans *et al.*, 1994; Lillycrop *et al.*, 2005; Taylor *et al.*, 2005). It is well-established that diseases in adult life such as hypertension and CVD and the subsequent development of the metabolic syndrome emerge as a consequence of interplay between genetic and environmental factors. Recent research has concentrated on the role undernutrition in pregnancy plays in the onset of disease. Whilst the origins of the metabolic syndrome are clearly multifactorial, nutrition in early life may have a profound influence upon risk and the responses of the individual to the environmental and lifestyle-related risk factors in adulthood. To date evidence has suggested that coronary heart disease (Langley-Evans *et al.*, 1998), hypertension (Langley-Evans *et al.*, 1996d), diabetes (Singhal *et al.*, 2003), obesity (Breier *et al.*, 2001) and impaired immunity (Beach, 1982) can be nutritionally programmed in early life and infancy. Previous work using protein restriction in rodent pregnancy has demonstrated its capacity to induce postnatal hypertension from as early as 4 weeks of age (Langley-Evans *et al.*, 1996d; Langley and Jackson, 1994) coupled with a reduction in nephron number from birth (Langley-Evans *et al.*, 1999a) within the F1 generation.

The primary aims of this thesis were to further explore the concept of fetal programming and ascertain whether the previously described phenotype of hypertension and reduced nephron number was passed to subsequent generations and to uncover the mechanism by which this phenotype is programmed *in utero* via

identifying key genes involved in this process. The results of this thesis support the original findings of Beach *et al* (1982) who noted that nutrient restriction during gestation had implications for future generations. Work within this thesis also confirms the existence of this previously described phenotype in the F1 generation and demonstrates that the phenotype is intergenerationally passed from the F1 generation to the F2 generation via both parental lines without any further dietary manipulation. This data effectively demonstrates that the course of an individuals development is not only influenced by a mixture of genetic, environmental and maternal factors but also grand-maternal and paternal factors.

It has been proposed that undernutrition during pregnancy programmes long-term changes in gene expression, which subsequently alter metabolism in the developing fetus resulting in abnormalities in later life (Burdge *et al.*, 2007a; Burdge *et al.*, 2007b; Waterland *et al.*, 2007). Indeed the expression of genes that either protect or predispose an organism against these conditions will be further modified by interactions between the genotype, early life nutrition and the postnatal environment (Langley-Evans, 2006).

A number of studies have indicated that prenatal undernutrition has the capacity to modulate the epigenetic regulation of gene expression (Burdge *et al.*, 2007a; Jaenisch and Bird, 2003). This raises the prospect that undernutrition can establish heritable changes to the epigenome and, as such, the disease programming effects of undernutrition in the fetal period may not be limited to the first generation. Emerging evidence from human and animal studies suggests that transgenerational effects may occur, whereby the consequences of deficits in maternal nutrition are subsequently passed on to the grandchildren (Beach, 1982; James, 2002; Pembrey, 1996). Indeed inheritance of epigenetic states is a common occurance in plants

(Takeda and Paszkowski, 2006). Specific epigenetic changes within tissues and especially gametes could play a vital role in mediating the transgenerational effect.

Unfortunately prior to commencing this study most mechanistic studies of epigenetics have focussed on F0 to F1 transmission, with very little evidence of transmission to further generations. There was, therefore, very little robust transgenerational work in the programming field. Early work had demonstrated that maternal diets moderately deficient in zinc resulted in a depressed immune function in the offspring which persisted until the F3 generation (Beach, 1982). Further to this Zambrano and colleagues noted that maternal protein restriction resulted in altered glucose and insulin metabolism in both the F1 and F2 generations (Zambrano *et al.*, 2005).

During the course of this study interest in the field of transgenerational effects and epigenetics has increased and several other research groups have also demonstrated evidence of intergenerational programming. Firstly Burdge and colleagues (2007a) demonstrated that after restriction of protein to the F0 generation PPARα and GR promoter methylation was significantly lower in F1 and F2 generation offspring. Similarly Pinheiro et al (2008) noted that protein restriction during gestation had adverse effects on both glucose and leptin metabolism, which resulted in insulin resistance in the F1 and F2 generations. Torrens and colleagues (2008) also recently demonstrated that maternal protein restriction during pregnancy leads to raised systolic blood pressure and endothelial cell dysfunction in both F1 and F2 generation offspring. All of these studies have one major limitation, they only assess maternal transmission, as they all use males from outside the study to breed the F2 generation and therefore do not conclusively demonstrate that these maternal traits are transmitted to the next generation via programmed influences on the ovum

as changes to the maternal environment and the composition of proteins in the developing embryo may play a key role. To date only a limited number of studies have demonstrated transgenerational programming in the F3 generation and beyond (Anway *et al.*, 2006b; Beach, 1982). Within the field of protein restriction Benyshek and colleagues (2006) demonstrated the F3 generation exhibited altered glucose metabolism (Benyshek *et al.*, 2006), although once again the research has limitations as animals from outside the experiment were used for breeding. To our knowledge the data contained within this thesis for the first time demonstrates that maternal protein restriction during gestation is able to programme both blood pressure and nephron number in both the maternal and paternal lines.

Current thinking is that the transgenerational passage of phenotypic information is likely to involve the epigenetic regulation of genes and to date several studies have demonstrated this principle, probably the most commonly cited is the work by Waterland and colleagues (2003) who have shown that dietary methyl supplementation of the diets of yellow agouti (A^{vy}) mice with folic acid, choline, vitamin B_{12} and betaine could alter the phenotype off the heterozygous offspring via increased CpG methylation at the Avy locus (Waterland and Jirtle, 2003). This effectively demonstrated that nutritional factors during fetal development can modify gene expression. Since this initial observation several other studies have also demonstrated epigenetic gene regulation such as Sinclair et al (2007) who fed sheep a diet deficient in methyl donors for eight days prior to conception and 6 days during pregnancy resulting in male animals with elevated blood pressure and insulin resistance, epigenetic analysis of these animals revealed that 4% of the 1400 CpG islands of the fetal liver monitored differentially methylated. Most of these CpG islands were either demethylated or hypomethylated (Sinclair *et al.*, 2007). These

studies are consistent with studies in the rat by Lillycrop and colleagues which also showed hypomethylation (Lillycrop *et al.*, 2005; Lillycrop *et al.*, 2008; Lillycrop *et al.*, 2007). Although within the current investigation no significant differences in DNA methylation were noted, a trend towards hypomethylation was apparent, which fits with prevailing epigenetic theories regarding the impact of epigenetics on gene expression. Although there is no doubt that epigenetic mechanisms such as DNA methylation and histone modification can impact upon the expression of genes and the development of disease, great care must be taken to ensure that these alterations are functionally significant. Studies by Lillycrop and colleagues (2008) have demonstrated that PPARα promoter was downregulated 26% in the offspring of rats fed a low protein diet during gestation whereas studies in the field of cancer suggest the methylation changes need to be of the order of two-fold or greater before they have a physiological impact (Veerla *et al.*, 2008).

The mechanism by which the low protein diet modifies epigenetic markers is still unknown. Figure 7.1 outlines the mechanistic arguments associated with the field of fetal programming and demonstrates how this thesis has advanced understanding of the development of disease in subsequent generations. Current thinking is that the expression of DNMT-1 maybe down-regulated (Lillycrop *et al.*, 2007). The availability of methyl donors such as methionine and folate is also thought to be of prime importance. Folate plays an essential role in the one-carbon metabolism pathway including the re-methylation of homocysteine to methionine, the precursor of S-adenosylmethionine, which acts as the primary methyl donor in DNA methylation (Kim, 2004). Experiments have demonstrated that methyl donor deficiencies have the potential to alter DNA methylation (Cravo *et al.*, 1992). Supplementation of the low protein diet with folate during rat pregnancy has been

demonstrated to prevent the establishment of the phenotypes associated with the low protein diet and normalise DNA methylation patterns (Lillycrop *et al.*, 2007; Torrens *et al.*, 2006). Recent research with in our lab has indicated that folate supplementation during pregnancy resulted in the overloading of the maternal system with folate. Most of this excess folate was stored in the maternal liver. Fetal tissues were also observed to have accumulated folate. Further to this no differences in hepatic global DNA methylation was noted in the offspring. Research within our lab has indicated that folate supplementation against a background of a control protein diet produced similar effects to the low protein diet alone (Engeham and Langley-Evans, 2009). This recent data suggests folate availability is not responsible for the programming effects of the low protein diet.

Although the field of transgenerational programming is fairly new, the process through which epigenetic mechanisms alter gene expression is fairly well understood. Two further fundamental questions remain. Firstly, the majority of the animal models in the programming field show that the effects of transgenerational programming peter out over time, whereby the F1 and F2 generations are affected but the F3 generation are unaffected. A possible explanation of this is that the initial stimulus modifies oocyte development in the F1 generation while they are still in the womb. Therefore the initial stimulus directly effects the development of the F2 generation. This suggests that there is not truly a transgenerational effect unless the effects of the intervention/ stimuli are seen within the F3 generation. However, this explanation can not explain findings in the current investigation as transmission occurs down both the male and female lines. The reason as to why the F3 generation is unaffected is unknown, however, it is possible that it is due to the loss of epigenetic marks laid down by the initial protein insult. Evidence from studies on

monozygotic twins suggest that the epigenome is constantly changing due to interaction with the external environment, independently of the genetic sequence (Fraga *et al.*, 2005). In particular, changes in epigenetic markers have been associated with aging and the formation of cancer (Fraga and Esteller, 2007). The ability of the epigenome to change over time indicates another possible explanation as to why the F3 generation is unaffected in the current investigation.

Secondly, it is not clear how epigenetic marks are transmitted intergenerationally as widespread reprogramming of the epigenome involving both active and passive demethylation and the reorganisation of histone modifications occurs during early development, followed by establishment of a different set of epigenetic marks associated with different cell lineages (Morgan *et al.*, 2005). Transgenerational programming could involve epigenetic marks that are resistant to this de-methylation process, or, it is possible that there is some sort of epigenetic memory which directs re-methylation. We propose that this is the most plausible cause of the transgenerational passage of the hypertensive and reduced nephron complement phenotype.

It must be noted that the epigenetic explanation offered within the current discussion is not the only viable mechanism as to how programming occurs. However, as previously stated, it is the most plausible. The glucocorticoid hypothesis and tissue remodelling may also play a part in the development of the phenotype associated with the low protein diet in the F1 generation, but they do not explain the transmission of the phenotype to the F2 generation.

The implications of the present findings for human health are vast. If maternal undernutrition is able to elicit major effects upon fetal development and the risk of disease then the field of programming is of major public health importance.

Furthermore if there is the possibility that these effects upon development and disease risk have the potential to be transmitted from one generation to another then research within this field is of paramount importance, especially for countries such as India and China where the rapid westernisation of the diet will result in a rapid and prolonged increase in the incidence of metabolic disease, unless there is appropiate intervention.

Epigenetic programming through the processes of DNA methylation, histone modification and chromatin remodelling is complex, involving numerous different mechanistic components and genes with altered transcriptional states. Before considering any interventions the processes involved must be thoroughly understood. Interventions may be as simple as improving the quality of the diet for pregnant women or may involve the administration of therapeutic agents such as folate or glycine which have been previously shown to cancel out the programming effects of the low protein diet (Jackson *et al.*, 2002; Torrens *et al.*, 2006). These methods would ideally work in combination with screening methods for the identification of the individuals at risk.

Epidemiological evidence from human cohorts and work from animal studies have clearly demonstrated that fetal programming by nutritional insults during gestation have huge implications for the risk of disease for several future generations. To date several different animals models have demonstrated their ability to induce common phenotypes that consist of CVD and development of the metabolic syndrome. Future work should concentrate on understanding the mechanisms associated with these processes as currently no single mechanism has been identified as being key to the development of these phenotypes. The identification of key genes involved in the different animal models would provide also provide a key insight into

the mechanisms involved. Future studies should also allow the separate consideration of the capacity of programmed male and female offspring to transmit their phenotype to subsequent generations by utilising both male and female animals from outside the experiment for breeding purposes. The analysis of epigenetic markers within the gametes, of programmed animals is also vital in order to understand the transmission of programmed phenotypes through successive generations. Analysis should be done using methylation-specific RT-PCR following bisulphite treatment and Chromatin Immunoprecipitation for the analysis of DNA methylation and histone acetylation.

In conclusion, it is clear that a nutritional protein insult during gestation has both physiological and metabolic implications for subsequent generations. Although understanding of the processes involved is limited, research into the role epigenetic mechanism play in the disease process are likely to be key in understanding how diseases are passed to subsequent generations. Only once the mechanisms and processes involved have been characterised can novel therapeutic strategies be invoked to combat the progression towards disease.

Figure 7.1 Possible mechanisms involved in the field of fetal programming.

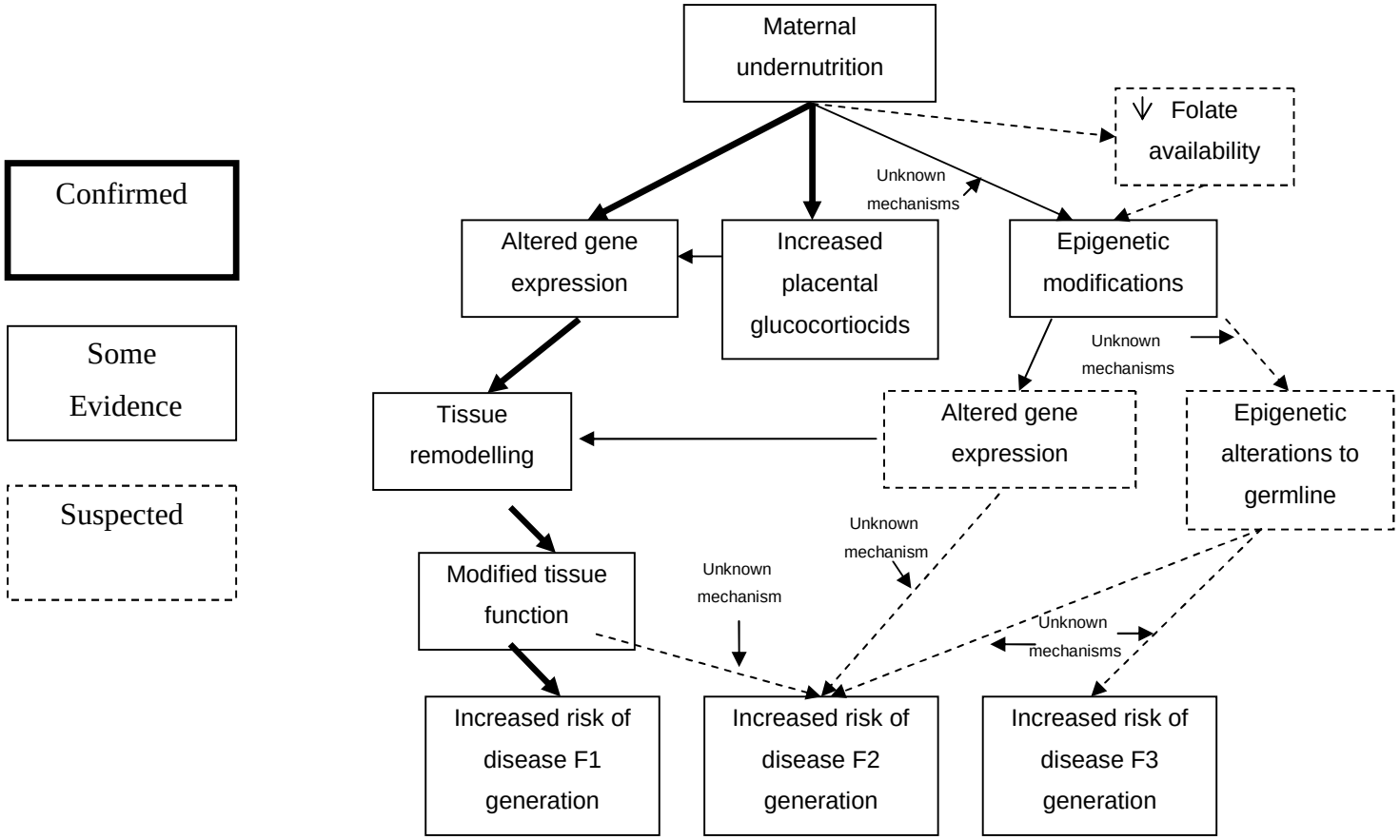

Figure 7.1 Maternal nutrient restriction is proposed to exert an influence on fetal development and the progression towards disease by either increased placental glucocorticoids and/or epigenetic modifications that ultimately alter gene expression and promote tissue remodelling, resulting in modified tissue function and the progression towards disease. It is suspected that epigenetic modifications to the germline have the potential to increase the risk of disease in subsequent generations.

8.0 Appendix

8.1 Addresses of suppliers:

Amersham Biosciences UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA
UK

Applied Biosystems Ltd
Lingley House
120 Birchwood Boulevard
Warrington
WA3 7QH
UK

Bio-Rad
1000 Alfred Nobel Drive
Hercules
CA 94547
USA

B & K Universal Ltd
Grimston
Aldbrough
Hull
HU11 4QE
UK

Crystal Chem Inc
1536 Brook Drive, Suite A
Downers Grove
IL 60515
USA

Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leicestershire
LE11 5RD
UK

Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park

Paisley
PA4 9RF
UK

Linton Instrumentation
Unit 11 Forge Business Centre
Upper Rose Lane
Palgrave
Diss
Norlfolk
IP22 1AP
UK

MWG Eurofins Operon
Anzingerstr. 7a
85560 Ebersberg
Germany

Perkin-Elmer
940 Winter Street
Waltham
Massachusetts
02451
USA

Promega UK
Chilworth Research Centre
Southampton
SO16 7NS
UK

Roche UK
Charles Avenue
Burgess Hill
West Sussex
RH15 9RY

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset
BH12 4XA
UK

Tecan Ltd
Tecan Way
Weymouth
Dorset
DT4 9TU
UK

8.2 Preparation of Solutions

8.2.1 Glucose Assay

0.1M Sodium Phosphate buffer
14.2g sodium phosphate in 1L H_20

Glucose reagent
Prepared using 100ml of 0.1M sodium phosphate buffer, pH 7.4 and adding 5mg glucose oxidase, 1mg horseradish peroxidise and 0.1g 2.2'azino-di-3[ethyl-benzthiazolin-sulphonate] (ABTS).

Glucose Standards
2mg/ml solution of glucose in sodium phosphate buffer.

8.3 Microarray gene information

Table 8.1 Microarray gene information

Accession Reference	Gene code	Gene
NM_017101	Ppia	Peptidylprolyl isomerase A
NM_012544	Ace	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
NM_001012006	Ace2	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2
NM_020306	Adam17	A disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)
XM_344544	Angpt2	Angiopoietin 2
NM_134432	Agt	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
NM_031009	Agtr1b	Angiotensin receptor 1b
NM_012494	Agtr2	Angiotensin II receptor, type 2
NM_012822	Alox5	Arachidonate 5-lipoxygenase
NM_053546	Angpt1	Angiopoietin 1
NM_013132	Anxa5	Annexin A5
NM_017059	Bax	Bcl2-associated X protein
NM_016993	Bcl2	B-cell leukemia/lymphoma 2
NM_133416	Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1
NM_031535	Bcl2l1	Bcl2-like 1
XM_226742	Birc1b	Baculoviral IAP repeat-containing 1b
NM_023987	Birc3	Baculoviral IAP repeat-containing 3
NM_022231	Birc4	Baculoviral IAP repeat-containing 4
NM_022274	Birc5	Baculoviral IAP repeat-containing 5
NM_053303	Blr1	Burkitt lymphoma receptor 1
NM_012762	Casp1	Caspase 1
NM_012922	Casp3	Caspase 3, apoptosis related cysteine protease
NM_031775	Casp6	Caspase 6
NM_022277	Casp8	Caspase 8
NM_031530	Ccl2	Chemokine (C-C motif) ligand 2
NM_019233	Ccl20	Chemokine (C-C motif) ligand 20
NM_031116	Ccl5	Chemokine (C-C motif) ligand 5
XM_226213	Cdh5_predicted	Cadherin 5 (predicted)
NM_057138	Cflar	CASP8 and FADD-like apoptosis regulator
NM_021655	Chga	Chromogranin A
XM_241632	Col18a1	Procollagen, type XVIII, alpha 1
NM_053617	Cpb2	Carboxypeptidase B2 (plasma)
XM_235061	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)
XM_340799	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
NM_017104	Csf3	Colony stimulating factor 3 (granulocyte)
NM_134455	Cx3cl1	Chemokine (C-X3-C motif) ligand 1
NM_030845	Cxcl1	Chemokine (C-X-C motif) ligand 1
NM_182952	Cxcl11	Chemokine (C-X-C motif) ligand 11
NM_053647	Cxcl2	Chemokine (C-X-C motif) ligand 2

NM_001012122	Ecgf1	Endothelial cell growth factor 1 (platelet-derived)
NM_012548	Edn1	Endothelin 1
NM_012549	Edn2	Endothelin 2
XM_345480	Edn3_mapped	Endothelin 3 (mapped)
NM_012550	Ednra	Endothelin receptor type A
NM_017333	Ednrb	Endothelin receptor type B
NM_022251	Enpep	Glutamyl aminopeptidase
NM_013057	F3	Coagulation factor III
NM_012846	Fgf1	Fibroblast growth factor 1
NM_019305	Fgf2	Fibroblast growth factor 2
NM_019306	Flt1	FMS-like tyrosine kinase 1
XM_221874	Flt3	FMS-like tyrosine kinase 3
NM_053652	Flt4	Fms-related tyrosine kinase 4
NM_019143	Fn1	Fibronectin 1
NM_138517	Gzmb	Granzyme B
NM_012967	Icam1	Intercellular adhesion molecule 1
NM_001007725	Icam2	Intercellular adhesion molecule 2
NM_019127	Ifnb1	Interferon beta 1, fibroblast
NM_133519	Il11	Interleukin 11
NM_013129	Il15	Interleukin 15
NM_031512	Il1b	Interleukin 1 beta
NM_031513	Il3	Interleukin 3
NM_012589	Il6	Interleukin 6
NM_013110	Il7	Interleukin 7
XM_235707	Itga5	Integrin alpha 5 (mapped)
XM_230950	Itgav_predicted	Integrin alpha V (predicted)
NM_017022	Itgb1	Integrin beta 1 (fibronectin receptor beta)
NM_153720	Itgb3	Integrin beta 3
NM_013062	Kdr	Kinase insert domain protein receptor
NM_022264	Kit	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
XM_001073384	Pla2g4c	Phospholipase A2, group IVC (cytosolic, calcium-independent)
NM_022214	Cxcl5	Chemokine (C-X-C motif) ligand 5
NM_031056	Mmp14	Matrix metallopeptidase 14 (membrane-inserted)
XM_235794	Mmp1a_predicted	Matrix metallopeptidase 1a (interstitial collagenase) (predicted)
NM_031054	Mmp2	Matrix metallopeptidase 2
NM_031055	Mmp9	Matrix metallopeptidase 9
NM_012611	Nos2	Nitric oxide synthase 2, inducible
NM_021838	Nos3	Nitric oxide synthase 3, endothelial cell
NM_031545	Nppb	Natriuretic peptide precursor type B
NM_012613	Npr1	Natriuretic peptide receptor 1
NM_031329	Ocln	Occludin
XM_214030	Pdgfra	Platelet derived growth factor receptor, alpha polypeptide
NM_031525	Pdgfrb	Platelet derived growth factor receptor, beta polypeptide
NM_031591	Pecam	Platelet/endothelial cell adhesion molecule
NM_001007729	Cxcl4	Chemokine (C-X-C motif) ligand 4
NM_053595	Pgf	Placental growth factor

NM_013151	Plat	Plasminogen activator, tissue
NM_013085	Plau	Plasminogen activator, urokinase
XM_574314	Plg	Plasminogen
NM_031557	Ptgis	Prostaglandin I2 (prostacyclin) synthase
NM_017232	Ptgs2	Prostaglandin-endoperoxide synthase 2
NM_138879	Sele	Selectin, endothelial cell
NM_019177	Sell	Selectin, lymphocyte
NM_013114	Selp	Selectin, platelet
NM_012620	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
NM_017050	Sod1	Superoxide dismutase 1
XM_342863	Tek	Endothelial-specific receptor tyrosine kinase
NM_017200	Tfpi	Tissue factor pathway inhibitor
NM_173141	Tfpi2	Tissue factor pathway inhibitor 2
NM_021578	Tgfb1	Transforming growth factor, beta 1
NM_031131	Tgfb2	Transforming growth factor, beta 2
NM_031771	Thbd	Thrombomodulin
XM_214778	Thbs2	Thrombospondin 2
NM_053819	Timp1	Tissue inhibitor of metallopeptidase 1
NM_012675	Tnf	Tumor necrosis factor (TNF superfamily, member 2)
NM_001024771	Tnip2	TNFAIP3 interacting protein 2
XM_344431	Tnfrsf10b_predicted	Tumor necrosis factor receptor superfamily, member 10b (predicted)
NM_012870	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
NM_139194	Tnfrsf6	Tumor necrosis factor receptor superfamily, member 6
NM_145681	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10
NM_012908	Faslg	Fas ligand (TNF superfamily, member 6)
NM_012889	Vcam1	Vascular cell adhesion molecule 1
NM_031836	Vegfa	Vascular endothelial growth factor A
XM_342759	Vwf	Von Willebrand factor
NM_017154	Xdh	Xanthine dehydrogenase
L08752	PUC18	PUC18 Plasmid DNA
	Blank	
	Blank	
SA_00005	AS1R2	Artificial Sequence 1 Related 2 (80% identity)(48/60)
SA_00004	AS1R1	Artificial Sequence 1 Related 1 (90% identity)(54/60)
SA_00003	AS1	Artificial Sequence 1
NM_013226	Rpl32	Ribosomal protein L32
NM_017025	Ldha	Lactate dehydrogenase A
NM_012495	Aldoa	Aldolase A
NM_012495	Aldoa	Aldolase A
NM_017008	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
NM_017008	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence
SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence

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