Feast or famine?
Altered maternal nutrition and disease risk in offspring

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Conference abstracts

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Sloboda DM, **Howie GJ**, Vickers MH. Maternal high fat nutrition either pre-conceptionally and / or throughout pregnancy and lactation leads to early-onset puberty in offspring and is further exacerbated by a post-weaning high fat diet. 5th International Congress on the Developmental Origins of Health and Disease, Perth, Australia, November 2007.

Sloboda DM, **Howie GJ**, Vickers MH. Early-onset puberty in offspring after maternal undernutrition is exaggerated by a post-weaning high fat diet: Sex specific evidence of nutritional mismatch. 5th International Congress on the Developmental Origins of Health and Disease, Perth, Australia, November 2007.

Symposium presentations

Howie GJ, Sloboda DM, Vickers MH. Alterations in the timing of maternal exposure to an obesogenic nutritional environment and obesity in male offspring – same metabolic phenotype, different underlying mechanisms? Science Symposium of the National Research Centre for Growth and Development, Auckland, New Zealand, August 2010.

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Abstract

Significant alterations in maternal nutrition may induce long-term metabolic consequences in offspring, in particular obesity and features characteristic of the metabolic syndrome. Although maternal nutrient deprivation has been well characterized in this context, little is known regarding the effects of maternal high fat (HF) nutrition on offspring.

Methods: The present study investigated the impact of two animal models of altered maternal nutrition on offspring metabolic phenotype: A) maternal calorie restriction, whereby pregnant dams were exposed to 50% undernutrition compared to controls during pregnancy, lactation or both, and B) maternal HF diet either pre-conceptionally and throughout pregnancy and lactation, or throughout pregnancy and lactation alone. Further, to examine the interaction between pre- and post-weaning diets on growth and metabolic outcomes, offspring were weaned onto either control or HF nutrition. Weight gain and body composition were recorded in mothers and offspring, and at postnatal day 180 blood and pancreata were collected from offspring. Importantly, both male and female offspring were examined to determine possible sex-specific altered susceptibility to metabolic disease.

Results: Maternal undernutrition imposed at different developmental windows resulted in offspring effects that were sex- and window dependant. Undernutrition during pregnancy alone, particularly when offspring were fed an unrestrained postnatal diet, resulted in offspring with increased adiposity and altered leptin sensitivity. Prevention of catch-up growth during lactation ameliorated the adverse metabolic effects associated with gestational undernutrition, particularly in females.

Adult offspring born to dams fed a HF diet during pregnancy and lactation, with or without a pre-conceptional HF diet, had similar obesogenic phenotypes, irrespective of post-weaning diet. This suggested that pre-conceptional obesity did not induce a phenotype different from the obesogenic diet during pregnancy and lactation alone. However, offspring of HF-fed mothers displayed differential pancreatic expression patterns of key genes in insulin and leptin signalling pathways, dependent on the window of exposure to the maternal obesogenic diet.

Conclusions: This study has provided novel insights into the differential effects of early life nutritional adversity during different critical windows of developmental plasticity on offspring growth and pancreatic leptin and insulin signalling. Possible mechanisms are discussed, together with a critique of the predictive adaptive responses hypothesis and the possible adverse impact of catch-up growth.

Contributors to this project

The size of this research project meant it was achieved only with the assistance of a considerable number of people, mainly from the Developmental Programming team at the Liggins Institute, University of Auckland, led by Dr Mark Vickers and Dr Deborah Sloboda.

The initiation and original design of the project belongs to Dr Mark Vickers. The bulk of the daily animal care and the routine weighing of rats and food intake were done by Graham Howie, with assistance from Tania Kamal. Dr Deborah Sloboda actively participated in the collection of the pubertal data. DEXA scanning under gaseous anaesthesia was performed by Graham Howie.

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The majority of the laboratory work was carried out by Graham Howie. Tania Kamal assisted with some of the insulin and leptin ELISAs. mRNA/DNA extraction, cDNA synthesis and amplification, and qPCR were all performed by Graham Howie. Kevin Dudley assisted with advice on primer design, and supplied the primer for insulin receptor (IR). Hong Liu performed the real-time PCRs for STAT3 and STAT5B.

Analysis of data, drawing of graphs and writing of this thesis was always done in the first instance by Graham Howie, with supervision, advice and correction from Drs Vickers and Sloboda. Prof Sir Peter Gluckman acted as consultant and advisor, and Dr Tony Pleasants offered statistical advice. Assistance with scientific style, final formatting and proofreading, was given by Dr Nina de Boo, Dr Ora Emslie and Mrs Helen Doherty.

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> Conceived and designed the experiments: MV. Performed the experiments: GJH TK. Analyzed the data: GJH MV DMS. Wrote the paper: MV GJH DMS.

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Conceived and designed the experiments: DMS MV. Performed the experiments: DMS GJH. Analyzed the data: DMS AP MV. Contributed reagents/materials/analysis tools: DMS AP PG MV. Wrote the paper: DMS PG MV.

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Names of dietary treatment groups:

CONT	=	Controls: standard chow diet from weaning and throughout pregnancy and lactation
UNP	=	50% undernutrition during pregnancy only
UNL	=	50% undernutrition during lactation only
UNPL	=	50% undernutrition during pregnancy and lactation
MHF	=	maternal high fat: high fat diet from weaning and throughout pregnancy and lactation
PLHF	=	pregnancy and lactation high fat: high fat diet during pregnancy and lactation only
-Chow	=	suffix indicating a post-weaning diet of standard chow
-HF	=	suffix indicating a post-weaning diet of high fat

Acronyms and initialisms for genes:

HPRT	=	hypoxanthine phosphoribosyltransferase
Ins1	=	insulin 1
Ins2	=	insulin 2
IR	=	insulin receptor
IRS1	=	insulin receptor substrate1
IRS2	=	insulin receptor substrate 2
K_{ATP}	=	ATP-sensitive potassium channel
ObRa	=	leptin receptor, isoform a
ObRb	=	leptin receptor, isoform b
PDE3B	=	phosphodiesterase 3B
Pdx1	=	pancreatic and duodenal homeobox 1
PI3K	=	phosphoinositide 3-kinase
SOCS3	=	suppressor of cytosine signalling 3
STAT3	=	signal transducer and activator of transcription 3
STAT5B	=	signal transducer and activator of transcription 5B

Other Abbreviations:

AD	=	ad libitum
AMPK	=	activated protein kinase
ANS	=	automatic nervous system
ATP	=	adenosine triphosphate
BLAST	=	Basic Local Alignment Search Tool
BMC	=	bone mineral content
BMD	=	bone mineral density
BMI	=	body mass index
BP	=	blood pressure
cAMP	=	cyclic adenosine monophosphate
CHD	=	coronary heart disease
C_t	=	crossing threshold
DEXA	=	dual energy Xray absorptiometry
DIO	=	diet-induced obesity
DOHaD	=	Developmental Origins of Health and Disease
ELISA	=	enzyme-linked immunosorbent assay

ERK	=	extracellular signal-regulated kinases
FFAs	=	free fatty acids
G6P	=	glucose 6 phosphate
GDM	=	gestational diabetes mellitus
GKB	=	glucokinase B
GLUT2	=	glucose transporter 2
GSIS	=	glucose stimulated insulin secretion
GTT	=	glucose tolerance test
HDL	=	high-density lipoprotein
HKG	=	house-keeping gene
HPA	=	hypothalamic-pituitary-adrenal
IGF	=	insulin-like growth factors
IGFBPs	=	insulin-like growth factors binding proteins
IHD	=	ischemic heart disease
IUGR	=	intrauterine growth retardation
JAK2	=	Janus kinase 2
LBW	=	low birthweight
LDL	=	low-density lipoprotein
NA	=	nose to anus
NAFLD	=	non alcoholic fatty liver disease
NLF	=	non-lactating females
NPY	=	neuropeptide- Y
NS	=	not significant
NT	=	nose to tail
NTC	=	no template control
OGTT	=	oral glucose tolerance test
PARS	=	Predictive Adaptive Responses
PIP3	=	phosphoinositide3,4,5 triphosphate
PTEN	=	phosphatase and tensin homolog
PVN	=	paraventricular nucleus
qPCR	=	quantitative polymerase chain reaction (ie, ‘real-time PCR’)
SEM	=	standard error of the mean
SH2	=	Src homology 2 protein domain
SPF	=	specific pathogen free
T2DM	=	type 2 diabetes mellitus
WAT	=	white adipose tissue

Chapter 1: Introduction

The introduction is divided into four major sections:

Part A Epidemiological evidence for developmental programming

A brief history of the origins of the DOHaD hypothesis and the early epidemiological studies of Barker and colleagues, plus the limitations of human population studies will be discussed.

Part B Animal models of developmental programming

A wide variety of animal models have been utilised for controlled empirical studies to probe the epidemiological data. Animal studies are essential to investigate the mechanistic links between prenatal and postnatal influences and the pathophysiological complications observed in later life. This section lays the theoretical base for the current project, which used a rodent model to examine the developmental programming phenomenon through manipulation of maternal nutrition during windows of development thought to be critical for influencing offspring phenotype. The interaction between maternal diet and post-weaning diet is also discussed (i.e. the concept of nutritional mismatch).

Part C Insulin, Leptin, & the Adipoinsular Axis

The normal activity of the pancreatic beta-cell (β-cell) is outlined, including glucose-stimulated insulin secretion. The action of leptin signalling pathways on insulin release is described, and the feedback interaction between insulin and leptin (the 'adipoinsular axis') is introduced.

Part D Obesity & Type 2 diabetes

Obesity and related metabolic disorders including Type 2 diabetes (T2DM) are growing public health concerns in developed countries and in developing societies transitioning to first-world economies, and one of our aims has been to shed light on the reasons and underlying mechanisms behind this phenomenon. This section examines the links between obesity and the development of T2DM.

Part A Epidemiological evidence for developmental programming

A1.1 Introduction: David Barker & the Hertfordshire cohort

Epidemiological studies provided the first evidence of links between early growth/development and subsequent adult disease states. Forsdhal, in Norway, was one of the forerunners. In 1977 he noticed the relationship between coronary artery disease and infant mortality rates in the 20 counties in Norway, and postulated that poor standards of living experienced in early life could lead to heart disease in adulthood (1). However, it was David Barker and colleagues from the University of Southampton who assumed the lead in this field, and they were instrumental in formulating the Fetal (later Developmental) Origins of Disease model, and establishing it as a credible area of research. So much so, that in its early manifestation it was known simply as the 'Barker Hypothesis'.

Looking back in 2003 David Barker described how his research group began their seminal investigations - "epidemiological studies of a kind never undertaken before" (p.1428) (2). The Barker group located a collection of birth and early health records of a large cohort of older people in the English county of Hertfordshire. Record collection had begun in Hertfordshire in 1911, largely due to the efforts of the first 'Inspector of Midwives', Ethel Margaret Burnside, and it continued through until 1948. The Hertfordshire epidemiological study traced 15,000 people from these original records, with birth dates prior to 1930, of whom about a fifth had already died. The causes of death were investigated, and those people still living were invited to participate in the study, through attending medical clinics for health assessment. By this means, an association between weight at birth (in particular low birth weight) and risk of disease in later adult life was uncovered. Describing these initial epidemiological studies, Barker wrote: "It seems remarkable that the weights of babies measured by Miss Burnside's simple spring balances, often in badly lit cottages and hovels, could so strongly predict events more than 50 years later" (p.1429) (2).

These were the epidemiological origins of the hypothesis that initially bore his name: "That undernutrition *in utero* permanently changes the body's structure, physiology, and metabolism, and leads to coronary heart disease and stroke in adult life" (p.13) (3). A whole series of studies was published out of Southampton from the mid 1980s, the earlier of which investigated links between low birth weight and high blood pressure or ischemic heart disease.

In 1998 Barker summarised the hypothesis in these words: “Poor nutrition, health and development … prejudice the ability of mothers to nourish their babies *in utero* and during infancy. The fetus responds to undernutrition with permanent changes in its physiology, metabolism and structure, and these lead to coronary heart disease and stroke [and diabetes] in adult life” (p.10) (3).

The advent of this new hypothesis was clearly an exciting time, and generated a whole raft of new ideas to investigate, test and evaluate. It provided a new angle of approach to adult conditions such as coronary artery disease and diabetes, where previous causal research had focused on adult life style factors such as diet or exercise. Areas such as fetal development and plasticity, paediatric endocrinology, maternal and fetal nutrition all received renewed attention. Around the world, other research groups sought to replicate and expand the first generation of epidemiological studies that came out of Southampton. Some of these international efforts are detailed below.

A1.2 Helsinki, Finland

The Helsinki studies were based on a peculiarly comprehensive set of registers that detail the lives of Finnish people: Birth Records; School Records; the Population Register (which allowed the current location of the birth subjects to be traced); the National Hospital Discharge Register (detailing diagnosis according to standardised criteria); the register of all people receiving long-term medication, maintained by the Social Insurance Institute; and the National Mortality Register (listing cause of death) (4-6).

The Helsinki cohorts, at around 3 to 3.5 thousand subjects, were larger than those in Hertfordshire. The Finnish data tended to support the British findings, and to extend them in many instances. There was collaboration between the two research groups: most of the Helsinki publications include Barker and Osmond from Southampton as co-authors. Similarly, when the fetal origins hypothesis was challenged, the Helsinki scientists responded in Barker’s defence (7). The Finnish group, like Barker’s team, published on various adult disease states but this overview will focus on studies into obesity and T2DM.

The fetal and childhood growth of persons who develop type 2 diabetes (2000) (5) examined diagnosed T2DM subjects among a population of over 7000 men and women, born between 1924 and 1933, whose birth measurements and early growth patterns had been recorded. The

proportion of T2DM sufferers rose as birth weight and birth length, ponderal index and weight of placenta all fell - a trend that was significant in men but not for women.

This study was hampered by the limitation of the historical data - no measurements were available for early childhood, the period between birth and 7 years. However, the data for the school years, 7-15, showed a marked difference between males and females who went on to develop T2DM. Having started small at birth both sexes had now 'caught up': boys had reached average size for 7 year olds, but the girls were above average. From then on both sexes exhibited faster growth rates than their peers. Among these faster growing children, the risk of developing T2DM was highest for those who had the lowest birth weights. For girls, an above average BMI was strongly associated with risk for T2DM. There was no such association for the boys. However, both sexes tended to have mothers who had a high BMI during pregnancy.

In 2002 the same cohort was utilised again (8). Rather than extracting data from registers and records, the Helsinki team invited a sample of the cohort, from among those still locally resident, to attend medical clinics where direct assessments of current health and body size could be undertaken. 474 people volunteered (176 men and 298 women).

The pattern for insulin resistance was different from the pattern uncovered earlier for T2DM. Both were associated with small size at birth, rapid growth in childhood (data available from 7 to 15 years only) and obesity in adult life, but the elderly subjects with insulin resistance had mothers with low BMIs during pregnancy, whereas the people with T2DM had mothers with high BMIs. The association of low maternal BMI with insulin resistance in these elderly offspring was powerful in itself, and independent of birth size or childhood BMI. Despite the differences in maternal BMI, many of the developmental characteristics leading to insulin resistance and T2DM were the same. This is consistent with the role of insulin resistance as a precursor of T2DM.

Two further studies from the Helsinki group investigated the growth rates in infancy and childhood of those adults who had developed T2DM (9, 10). Another cohort was used, born 1934-1944, where there were records of growth throughout the first year of life and continuing to 12 years of age. An association was uncovered between T2DM and early adiposity rebound. Adiposity rebound is the age at which BMI rises again in young children, after a period of normal decrease. In Western children the typical pattern is a fall in BMI from about 2 years of age and then a rise again after about 6. However, babies born small and who remain small at 1 year experienced an early adiposity rebound, and this pattern of growth was associated with the development of T2DM later in adult life (9).

Another pathway of growth that could lead to T2DM was seen in above-average birth weight babies who suffered poor growth in the early postnatal period. The authors speculated that faltering of growth in the first 3 months of life caused an alteration of insulin metabolism that persisted throughout life (10).

A1.3 Other cohorts

South India: The Southampton team were also involved in studies undertaken in India, using birth records preserved in hospitals from the 1930s – 1950s. India would seem to be an ideal place to conduct such studies, given that some newborns might be expected to be underweight due to undernutrition, while rising standards of living were also altering diet among certain classes of society, and the incidence of obesity, coronary artery disease and T2DM were on the rise.

Links between coronary heart disease and birth size were reported in 1996 (7). In terms of diabetes, the South India studies also purported to show that intrauterine programming led to Type 2 diabetes (11, 12). However, the detail of fetal programming was different from previous studies: in Europe and the USA, development of T2DM was associated with low birth weight or thinness at birth with a low PI; whereas in South India it was shortness at birth and a high PI, plus an above average maternal weight during pregnancy that led to T2DM.

Sweden Extensive record-taking of the population is the social norm in Sweden, and sizeable cohorts of men and women have been raised from the various records and registers to test for associations between measurements recorded at birth and later development of adult disease states (13-17).

An association between low birth weight and the development of obesity in adult life was demonstrated (15), plus a similar association with T2DM (17).

A1.4 Conclusions of the 'first generation' of epidemiological studies

A decade or so after the original Southampton studies began to appear and the 'Barker Hypothesis' was first formulated, it appeared as if the link between fetal nutrition and development of adult diseases had reached widespread international acceptance. In 2001, in a review of the epidemiological data, Hales & Barker confidently asserted: "The validity of the findings is now generally accepted" (18). They listed 32 studies from a variety of countries in support of the association between small size at birth and the metabolic syndrome. Indeed they stated, "We are not aware of any study that contradicts [these findings]" (18). The focus of their paper then proceeded to discuss the possible mechanisms underlying programming, and to offer directions for further research. One of the areas proposed for further investigation was how intrauterine undernutrition interacts with factors arising in later life (during infancy or childhood or adulthood) to produce adult disease states. Some of those interacting factors were already known (for example, rapid weight gain in childhood, and obesity in adult life) – it was merely the architecture of the interaction that required further delineation. This review by Hales and Barker was founded on the premise that the hypothesis was established and unshakeable.

A2 Non-nutritional factors influencing birth weight

A2.1 The challenge to the fetal origins hypothesis

A challenge to the first generation of epidemiological studies was raised by Rachel Huxley and colleagues, beginning in 2002, in articles with provocative titles such as 'Unravelling the fetal origins hypothesis' (19) and 'Fatal flaw in the fetal argument' (20). Huxley is herself an epidemiologist and the 2002 challenge was a meta-analysis examining the association between birth weight and adult blood pressure.

Interestingly, Huxley had been the lead author only 2 years earlier (other authors were members of the Southampton group), of a very similar meta-analysis concluding that an inverse relationship between birth weight and systolic BP did truly exist (21). In the intervening period Huxley had clearly begun to question the validity of the data and her previous conclusions. The new review asserted that the observed association could well be erroneous and simply due to confounds, bias and other methodological mistakes.

A2.2 Confounds

Huxley's argument was that other factors were confounding the fetal origins hypothesis, and that these confounds – in particular, socioeconomic status – could alone account for the observed association with increased risk of adult disease. Huxley *et al.* pointed out that socioeconomic status has impacts on diet choice, physical activity and exercise, health care, incidence of smoking; any of these during pregnancy could potentially affect birth weight of the baby.

Epidemiological studies are typically observational rather than experimental, which makes it difficult to control for all variables and thus admits the possibility of unrecognised confounds. Barker himself accepted this. In Epidemiology for the Uninitiated, a textbook co-authored by Barker, he wrote: "If confounding influences are identified in advance then allowing for them in the design and analysis of the [observational] study may be possible. There is still, however, the chance of unrecognised confounders" p.51, (22). Later, in a section entitled Confounding *versus* Causality he observed: "Assessment of whether an observed association is causal depends in part on what is known about the biology of the relation" p.67, (22). Identifying the biological mechanisms underlying the developmental origins claim would help cement causal links, dispersing doubts about potential confounds. The thing about confounds is that they in their turn must stand up to scrutiny, and must offer a sufficient alternative explanation for any observed association. "If an association is to be explained by confounding then the confounder must carry

an even higher relative risk for the disease and also be strongly associated with the exposure under study" p.68, (22).

Huxley *et al.* raised valid points regarding sociodemographic status. The socioeconomic status of the parents is likely to strongly influence the nutritional circumstances of the baby, both during pregnancy and after birth. However, the Hertfordshire data did not contain any information about the socioeconomic circumstances of the family, and the Helsinki data was limited to father's occupation, plus number of people in the family and number of rooms in the family dwelling, which was used to calculate 'overcrowding', i.e. numbers of people per dwelling, taken as another indicator of socioeconomic status (10). These did not yield particularly fine-grained statistics however, since fully two thirds of the cohort were defined as 'labourers' and 'lower social class', although the authors argued that this was consistent with the population of Helsinki at the time. They found no link between post-natal growth and overcrowding.

A counter argument is the fact that malnutrition appears to make surprisingly little difference to the developing fetus. It seems that the mother's homeostasis and the placenta act as buffers against a variable external world, protecting the supply line to the fetus, and generally maintaining an optimal and steady state nutritional environment for the baby, except for extreme situations. For example, in the Dutch Famine Winter, which lasted over seven months, from September 1944 to May 1945, only those babies that had suffered undernutrition during their third trimester were born small. Those who were exposed to undernourishment during the first and second trimesters only, then adequately fed in the last third of pregnancy (after food supplies were restored by the Allied liberation) were born with normal birth weights (23, 24).

I will now examine and discuss the various proposed confounds that might potentially account for the purported link between birth weight and adult disease states.

A2.3 Smoking during pregnancy

Maternal smoking during pregnancy is well documented to have adverse effects on birth weight (25). It has been estimated that smoking during pregnancy may account for 21-39% of all cases of low birth weight (26). In a Brazilian cohort where nearly a quarter of the mothers smoked throughout pregnancy, babies born to smokers weighed 4.5% less than babies born to non-smokers, with an odds ratio for low birth weight of 1.59. Intrauterine growth retardation (thought to be the prime cause of the lowered birth weights) was dose-dependent on the number of cigarettes smoked, and was also witnessed in babies of non-smoking mothers who had smoking partners (27). Similar results have been seen in European cohorts (28-30). Neither the

Hertfordshire nor the Helsinki data sets contained information on whether or not mothers smoked during pregnancy or whether or not they were exposed to environmental ('passive') smoking. Thus, maternal smoking, known to affect birth weight was an uncontrolled-for variable, and a potential confound in these studies.

A2.4 Socioeconomic status

Low birth weight babies are more common among lower socioeconomic groups (31-35). Barker's early studies arose out of exactly such an observation - that prevalence of adult IHD (ischemic heart disease) geographically matched prevalence of infant mortality in UK local authorities (36). Thus this 1986 paper speculated that poor living conditions along with poor nutrition (i.e., low socioeconomic status) set the conditions for later development of IHD. However, despite the widespread evidence for and the general acceptance of a link between poverty and low birth weight babies, the underlying mechanisms are still not clearly delineated. There may be biological factors such as the weight, age, parity of the mother, or interval between pregnancies. Or environmental factors may predominate, such as smoking or exposure to other toxins/pollution (e.g. in a work or living environment), domestic or occupational stress, increased exposure to infectious illnesses, or underutilisation of services for antenatal care. (Smoking is thought to be the largest of these contributors (25).) All these factors may work in combination, along with psychosocial factors that affect a woman's self-image, her attitude toward herself and her lot during pregnancy, and together these may produce an enduring 'culture of poverty' (32, 37, 38). Referring to individual women, Kramer *et al.* called this a 'depressive self-concept': it is a vicious cycle that generates stress and in turn is reinforced by stress (32). Hart argued that it was the historical trans-generational legacy of good nutrition and well-being that allowed women exposed to the Dutch Hunger Winter to produce healthy babies during a period of severe deprivation (39). She saw this in cultural socioeconomic terms: "the cumulative legacy of a society's own developmental history, deeply rooted in institutional norms and the early material experience of both current and preceding maternal generations" (p.29). Sparen *et al.* (2003) claimed that lifestyle and socioeconomic factors played no role in their finding of raised BP associated with the famine of the Siege of Leningrad (40), although their findings were confounded by the effects of stress and war-time trauma (41).

'Socioeconomic status' is not simple to accurately quantify; it is defined by selected indicators or proxies of status. A great variety of these have been utilised, and they differ, appropriately, according to culture and country. Typical measures commonly used in Western countries include: occupation, household income, educational attainment, house ownership (or type of house),

marital status, receipt of social welfare. In the USA, race was once used, given that social inequalities were historically so tightly bound to race – an assumption that ignored any possibility of heterogeneity. In one recent study examining factors contributing to low birth weight in Mexico, the authors found two proxies of socioeconomic status to be most reliable – employment, and ownership of certain goods, i.e. a dwelling and a car (42). These authors reached a conclusion that could be echoed by many of these studies: "Although many socioeconomic factors related to LBW [low birth weight] have been identified, the specific role of each of them is not known, limiting the ability to use preventive actions in exposed populations" (42). Socioeconomic status is multi-factorial and culturally defined, and this presents difficulties in understanding its connection and association with low birth weight babies.

Nevertheless, socioeconomic status is a confound for the developmental origins hypothesis: there is indeed a consistent direct association between low socioeconomic status and low birth weight; thus it could be argued that development of the metabolic syndrome in adult life is really due to socioeconomic conditions during pregnancy and early life. Barker's emphasis on nutrition could conceivably be mistaken because nutrition is only one specific aspect within the broader multi-factorial construct of lower socioeconomic status. It could be that some other socioeconomic factor (or combination of factors) has a stronger effect on the fetus and neonate, programming for disease states in adulthood. The only way to resolve this question is to determine the mechanisms underlying the association.

Harding (43) has argued that nutrition is the most likely candidate for the programming mechanism. She cites three lines of evidence: firstly, animal studies where IUGR is readily produced by manipulating fetal nutrition, leading to hypertension and glucose intolerance; secondly, 'natural experiments' in human populations, such as the Dutch Hunger Winter; and thirdly, the known biology of fetal growth within the intrauterine environment – how growth is regulated by insulin and insulin-like growth factors (IGF), which are in turn tuned to the fetus's nutritional supply. Thus fetal development is modulated by nutrient supply and an altered hormonal environment, and the two are linked. Harding does not discuss the other endocrine axis that is thought to be relevant: the hypothalamus-pituitary-adrenal (HPA) axis.

A2.5 Stress & the HPA axis

The HPA axis is one of the major hormonal systems that mediate the stress response. (Another major system involved in stress is the Autonomic Nervous System, the ANS.) Subjecting pregnant rats to stress increased their levels of corticosterone, in both dams and fetuses, with two results: firstly, the HPA axis was programmed so that offspring had altered responses to stressful stimuli in later life (44). Interestingly, these changes differed markedly with sex (45). Secondly, these excess steroid-exposed fetuses were born with low birth weight and went on to develop hyperglycemia and hypertension, i.e. the metabolic syndrome (46-48). It may be that similar mechanisms are involved, or they may be distinct. The HPA axis changes can be induced by the stress of undernutrition (49, 50).

Which is the primary mechanism, the undernutrition or the HPA axis changes? Kanaka-Gantenbein *et al.* assert, "Intrauterine growth retardation is usually due to maternal, fetal factors, or placental insufficiency, while endocrine factors represent just a small minority in its etiology. Main endocrine-related causes of IUGR are disorders in insulin and insulin-like growth factor-1 (IGF-1) secretion or action" (51).

A2.6 Maternal height

Height is another variable that is directly related to socioeconomic status (33, 52). Well-to-do people tend to have taller offspring than the poor, for reasons that are not well understood, although dietary differences are suspected as being a main contributor. Some dietary differentials have been identified: the highest amounts of fat consumption were seen in households where the main source of income was manual labour (53); parents with lower educational attainment had greater pre-pregnancy BMIs and were more likely to be overweight than those with higher education; moreover, they showed greater weight gain during pregnancy (54). After birth, children born of lower socioeconomic parents tended to eat more sweets and crisps, and drink more soft drinks (55); they received less fibre, i.e. fruit and vegetables (56). All this evidence suggests that there are real dietary differences between lower *versus* higher socioeconomic groups, which may contribute to the linear growth and the eventual height of the offspring.

A2.7 Controlling for confounds

Many of the confounds that challenge the developmental origins hypothesis are due to the retrospective nature of the early epidemiological studies, and the inability of that approach to control for all variables.

Therefore, more recent human epidemiological studies, being cognisant of these challenges, have attempted to include data on potential confounds. For example, Hardy *et al.* (57) found that socioeconomic class, mother's educational attainment, mother's height – none of these confounded the association between low birth weight and adult BP. They noted, however, a birth order effect (firstborns tend to have higher systolic BPs as adults), and a maternal age effect (adults with higher BPs tended to be born to younger mothers). They suggested that both these effects might be caused by stress mediated via the HPA axis.

Another study by Hardy's team (58), specifically citing Huxley *et al.*'s challenge, gathered information on: maternal diet and physical exercise; maternal age; pre-pregnancy weight; birth order of the fetus; maternal height; smoking; socioeconomic status. The authors reported little consistent effects from most confounders. The association between birth weight and adult BP was weakened when adjustment was made for adult BMI, but accounting for socioeconomic factors, maternal smoking and diet, birth order – none of these factors made appreciable differences to the association. The only two factors highlighted were gestational age (prematurity as opposed to simple birth weight) and "possibly maternal pre-pregnancy weight" p.29, (58). Other studies which have found little influence from socioeconomic factors include (59, 60).

The American 'Nurses' Health Study' (61, 62) and the American men's 'Health Professionals Follow-Up Study' (63)both attempted to overcome the problem of confounds. The Nurses' Health Study enrolled an impressive sample size of >71,000 women, and attrition rates of less than 10% were reported. However the women's data was based on a postal questionnaire, not on actual clinical measurements. Despite testing undertaken to validate the data, the criticism remains that the subjects were all self-selected, were confined to a single socio-demographic group, namely, registered nurses, and that both birthweight and blood pressure were self reported. The underlying assumption was that health professionals would necessarily report this kind of data accurately and honestly. Yet when a sample of responses concerning birthweights was compared to actual figures recorded on birth records, the concurrence rate was only 70% (64).

An attractive alternative to the complications of human epidemiology are studies utilising animals. Animal studies typically involve much shorter timeframes and provide the opportunity to manipulate environmental variables, thus controlling for confounds.

A3 Conclusions:

1. Influences other than the fetal environment are now also acknowledged, e.g. the pre-conceptional and neonatal/postnatal nutritional environments – hence the progression of the name from Fetal Origins to Developmental Origins. These findings and the hypothesis have survived the rigorous challenges of Huxley *et al.* and others (19, 20, 65, 66).

2. The bulk of the epidemiological studies are based on retrospective historical data – people born in the early part of the century or mid-way through it. The question remains whether these data apply to today's situations, today's populations. Generational differences could include: different patterns of breastfeeding and weaning; different formulae for artificial milks in bottle-fed babies; different burdens of infection; different family sizes; and mothers in earlier generations would likely have less income and economic power, and less education (67). However, numbers of prospective studies are underway (68-71).

   If, as the developmental origins model suggests, it is the transition from one nutritional state to another that increases the risk of developing certain diseases in adulthood – whereas continuity and steady-state nutrition across generations seems to be protective against development of the same diseases – then it may be possible that the developmental origins phenomenon only applies in certain historical settings/conditions, i.e. when societies are in nutritional transition, as the West was in the early 20th century, and as many non-Western countries are currently. Thus, closely monitored cross-cultural prospective studies are needed.

3. Birth weight is only a snapshot, a singular measure on a continuum of growth, a proxy for prenatal growth and development. Early postnatal nutrition, particularly during the first year of life (so-called catch-up growth) is a continuation of the period of sensitive plasticity where physiological set points can be fine tuned, the physiology that underlies obesity, high blood pressure, atherosclerosis and type 2 diabetes. Critical periods may extend even beyond the early years. Barker himself says there are three critical periods for

entraining body weight – prenatal, childhood (where adiposity rebound occurs), and adolescence (3). Thus, a whole of life perspective is necessary to understand the development of the metabolic syndrome.

4. A life course perspective sees intrauterine undernutrition as only one factor, one contributor, leading to the metabolic syndrome. Other components include well-known adult lifestyle factors such as lack of exercise, obesity, high cholesterol diet, smoking, etc. In public health terms, tackling conditions such as adult hypertension is probably better achieved by addressing the lifestyle factors, because as Ruth Morley writes: "A major issue is our inability to modify birth weight to any relevant extent. Associations are expressed in terms of the 'effect' of a 1kg change in birth weight. To date no intervention in the human has influenced birth weight to anything approaching this extent" (p.75) (72). Moreover, management of adult hypertension is pharmacologically achievable, relatively easily and readily done, and perhaps more cost effective. In terms of the developmental origins hypothesis, public health money should first be invested in research to determine causality, and on those aspects of fetal and infant/child development which can be shown to be directly linked to adult disease and which are realistically modifiable.

5. It is impossible to unravel the whole picture through epidemiological studies alone, because of their inability to control for all variables. For example, it is impossible to monitor every human subject's food intake, or measure stress levels through each trimester of pregnancy. Epidemiological studies are limited, which is why animal studies are necessary. The standardised conditions that are possible in studies with animals will eliminate many of the confounds that bedevil the human epidemiological data.

   Animal models of under- and overnutrition need to be tested and validated, before the findings can be applied to humans. It is noteworthy, however, that the amount of weight change in the rat fetus achieved by dietary manipulation far exceeds what is typically seen in humans, even in settings of extreme famine. This may limit the value of rodent modelling for understanding the fetal supply line in humans. Hales and Barker commented on this: "While the use of reduced maternal protein intake as an experimental model in animals may not reflect the commonest problem facing human populations, it may evoke common fetal response to undernutrition. This may explain why this specific and limited nutritional deficiency induces a phenotype with such remarkable parallels to the human metabolic syndrome and type 2 diabetes" p.14, (18). The testing and validation of new nutritional models in rats is one of the prime purposes of this thesis.

Part B: Animal Models of Developmental Programming

B1.1 Introduction

The limitations of clinical studies has made development of animal models an important means of assessing the role of nutritional status on pregnancy and the health and well-being of offspring. Evidence from animal studies has been widely cited in support of the programming hypothesis (73). Animal models have been developed in many species including rodents, sheep and guinea pigs with various insults to induce developmental reprogramming (global undernutrition, low protein, high fat, uterine artery ligation, glucocorticoid exposure etc). The rat is the most widely used species to model altered maternal nutrition and the impact on the health of offspring. Despite the obvious limitation of using an altricial species to model observations in a precocial species, the phenotypes observed in the rat models closely parallel that seen in the human setting, particularly as relates to the development of the metabolic syndrome.

Animal work allows the investigation of the mechanisms that underlie developmental programming – for example, the relative importance of intrauterine conditions *versus* postnatal conditions; the critical windows during fetal development when perturbation sets conditions that lead causally to manifestations of disease in later life; the cellular and systems-wide underpinnings of these processes.

Several models of altered maternal diet have been established, all leading to varying degrees of developmental programming of offspring. Examples of maternal undernutrition models include the reduction of specific dietary nutrients, such as in the low-protein model, or the iron deficiency model; and the global reduction of all dietary nutrients, either by restricting nutrient supply or by means of uterine artery ligation. At the other end of the spectrum is the increasing prevalence of maternal obesogenic models including high fat (HF) diets such as the ‘cafeteria diet’, which is high in fats and sucrose (74-76). The phenotypes of offspring at both ends of the maternal nutritional spectrum, i.e. under- *versus* over-nutrition, are remarkably similar; for example, there is a “U” shaped curve in the relationship between birth weight and obesity (61, 77). The current project therefore examined two models of maternal nutrition: a global undernutrition model, and a dietary HF model. Thus there were two main divisions to this study – a maternal undernutrition experiment (Chapter 3), and a maternal HF experiment (Chapter 4). In addition, to examine the mismatch hypothesis of Gluckman and Hanson (78, 79), we also studied the interaction between

maternal and postnatal diets via placement of offspring on two levels of post-weaning nutrition: standard chow or a HF diet.

A closer examination of these various rodent models of developmental programming now follows, with a greater emphasis on the models employed in the current study.

B1.2 Various animal models

The low-protein model

The maternal low-protein model is one of the most widely used models in the rat to induce developmental programming and has been shown to effectively replicate many features of adult onset insulin resistance and T2DM. Typically dams are fed a diet that contains half the protein content of Controls (9% *versus* 18-20%). Protein deficiency during gestation produced offspring that were small at birth, and developed hypertension in adulthood, cardiovascular disease and progressive decline of kidney function (80-85). Extending the low-protein treatment through the lactation period led to permanent growth restriction, even if offspring were returned to a normal diet at weaning (86). Catch-up growth was evident if lactational nutrition was unrestrained, and such accelerated weight gain contributed to deteriorating insulin sensitivity in adulthood (87, 88). Offspring of low-protein mothers had reduced pancreatic islet mass and vascularity (89), and impaired insulin release (90), all of which could contribute to insulin resistance and/or diabetes in adulthood. Hepatic response to insulin was also affected (91) Interestingly, administration of leptin to low-protein dams during pregnancy and lactation conferred protection to offspring against the development of obesity and T2DM in later life, although the effect of leptin treatment to control dams was not investigated (92).

The mechanism underlying the low-protein insult is thought in part to be insufficiency of essential amino acids, for example, taurine. Administration of supplementary taurine to low-protein dams during gestation and lactation prevented some of the pancreatic developmental deficits normally seen in such programmed offspring (93). In addition, methyl donor supplementation with folic acid reversed the hypomethylation of key metabolic genes in LP (low-protein) offspring, and maternal supplementation with glycine and folic acid prevented the development of hypertension in LP animals (94, 95). Work by Langley-Evans also showed that the methionine content of the low-protein diet can play a major role in the progression to hypertension (96, 97).

Maternal iron restriction

The maternal iron restriction model grew out of the initial epidemiological studies, based on data that was originally concerned with anaemia in human pregnancies and its effect on the developing fetus (98-100). Deficiency of iron in the maternal diet led to low birth weight pups, with reduced haemoglobin levels and increased myocardial weight at weaning, and elevated BP in maturity (98). The same rodent model was used to investigate the effect of maternal iron deficiency on insulin secretion and resistance, but insulin concentrations were unchanged and glucose tolerance improved in the treatment offspring (101). These measurements were obtained at age P90 and it was possible that they would have deteriorated with age. However, a follow-up study found no change at 16 months of age (102). Thus, the maternal iron restriction model has some similarities with other maternal nutrition deficiency models (an association between low birth weight and hypertension), but appears to affect cardiovascular physiology more than metabolic indices.

Uterine artery ligation

Uterine artery ligation is an attempt to model or replicate uteroplacental insufficiency, thought to be a major cause of intrauterine growth retardation (IUGR) in humans. The uterine artery in the rat is tied off on one side only, leaving the nonligated uterine horn to serve as a control. With the uterine artery unilaterally closed, the sole blood supply to the affected side is via the ovarian artery (103). Typically this intervention is performed late in gestation, G17-G19, during the period of rapid fetal growth. Offspring from this preparation were typically born growth retarded. They exhibited altered hepatic glucose metabolism early in life and went on to develop frank diabetes (104), although gender effects have been reported with females developing glucose intolerance ahead of males (105). Muscle uptake of glucose and stored glycogen levels were also impaired (106). (NB: Both uterine arteries are ligated in some uterine artery ligation models.)

Of relevance to the current study, Park *et al.* 2008 found that IUGR following uterine artery ligation led to progressive epigenetic silencing of expression of the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) in β-cells of offspring. They proposed this as the mechanism underlying the development of diabetes in adult IUGR offspring (107). Furthermore, they were able to reverse the abnormalities associated with IUGR via treatment of offspring during the neonatal period with the GLP-1 analogue Exendin 4 (108).

We also investigated the relative expression of the Pdx1 gene in offspring of our maternal HF diet model. Pdx1 is critical for β-cell development and maintenance and we hypothesised that expression levels of Pdx1 would be altered in offspring of dams exposed to HF dietary insult.

B1.3 Global dietary restriction (undernutrition)

Restriction of food supply during pregnancy leads to reduced nutrient supply and decreased placental blood supply to the fetus (109). There has been extensive modelling of prenatal dietary restriction in rodents. Maternal nutrition has been manipulated during different stages of gestation, and the results have varied depending on the timing of the restriction, the length and degree of the undernutrition.

Various models of global dietary restriction have been employed, ranging from moderate to severe:

- 30% reduction of the caloric intake of controls (110, 111)
- 50% reduction (112) (113)
- 70% reduction (114, 115)

Ozaki *et al.* 2001 (110) describe their regime of 30% dietary reduction during days 0-18 of pregnancy as 'moderate'. The level of nutrition was determined from an earlier pilot study of pregnant control animals. Pups were born IUGR, but caught up with control offspring within 20 days. As adults they developed higher blood pressure (BP) compared to controls, and a gender effect was evident – the hypertension developed earlier in the males than the females. Yura et al. (2005) also applied a 30% undernutrition, from day 10 of gestation until parturition. This intervention produced reduced birth weight pups that experienced catch-up growth leading to diet-induced obesity in adult life (111).

Thus, a reduction of only 30% of maternal food intake is capable of programming elements of the metabolic syndrome. Neither of the groups cited above measured percentage body fat; thus adiposity was assessed by body weight and/or isolated fat pad mass, relative to Controls. Both groups reported normoglycemia but neither study assessed insulin sensitivity.

It is noteworthy that this mild restriction of just 30% can elicit a programmed phenotype because it has been argued that feral rats in the wild exist on a diet that is no more abundant - just 70% of the *ad libitum* calories consumed by laboratory control rats (116, 117). Perhaps laboratory control rats are not true controls after all, perhaps they are simply 'laboratory gluttons' (Austad's phrase, (117)). However, 30%UN reliably engendered developmental programming in these studies, a similar effect to that seen in other models of undernutrition. Moreover, measurements of feral rodent food consumption have demonstrated that laboratory rats do not eat amounts in excess of animals in the wild (118).

Holemans *et al.* (1999) used a model of 50% undernutrition from days 11-23 of pregnancy (113). They applied this treatment during pregnancy alone *versus* pregnancy and lactation. Neither group of offspring exhibited any changes in BP or heart rate at age 100 days. There were subtle changes in vascular function, and mild levels of hyperglycemia and hypoinsulinemia.

The pups which were exposed to 50% maternal undernutrition during pregnancy alone were born small, but when they received normal lactation and an *ad libitum* post-weaning diet, their growth and body weight surpassed the Controls. This is a typical example of neonatal 'catch-up' growth. By contrast the pups that were undernourished through both pregnancy and lactation were born small and remained small (119).

The 50% undernutrition model used by Holemans *et al.* (113, 119) failed to produce the expected programming of blood pressure. This was probably related to the window of exposure to maternal undernutrition, or it may be due to the small numbers of animals used. The study also utilised female offspring only, so no gender effects were examined. The small but significant effect on blood glucose regulation is noteworthy. Likewise this group demonstrated increased insulin resistance in adult females utilising the 50%undernutrition model (119).

Bertin *et al.* (2002) applied a 50% undernutrition model and observed a significant reduction in pancreatic β-cell mass in fetuses at age 21.5 days of gestation (120). However, maternal nutrition was restricted only through the last trimester of pregnancy, and offspring exhibited undisturbed glucose metabolism in adulthood. By contrast, a 50% undernutrition regime applied not only in the last trimester of pregnancy but also throughout lactation, produced offspring with decreased ability to manufacture insulin, and with altered glucose tolerance (121). This suggests that the sensitive window for pancreatic development extends beyond gestation into neonatal life.

An even more "severe" undernutrition model was used by Woodall *et al.* 1996 (115), just 30% of the amount consumed by control dams *ad libitum*. At this level of restricted maternal nutrition, pups were significantly growth retarded, and placenta size was reduced. Litter size, however, was unaffected. During lactation (whereby dams were returned to *ad libitum* food supply) there was no difference in nursing behaviour, nor any incidence of cannibalism, but there was an increased pup mortality within the undernourished group. The IUGR pups never caught up to the body weight of the controls when fed a standard diet. Blood pressure was measured at 30 weeks and 56 weeks of age, and the undernourished cohort exhibited programmed hypertension.

Woodall *et al.* (115) again used the 30% undernutrition model to investigate the role of insulin-like growth factors (IGFs) and the associated IGF binding proteins (IGFBPs) in fetal development. They found the endocrine parameters of the somatotrophic axis had been changed, in both the dams and the offspring, following undernutrition.

Vickers *et al.* 2001 (114) also used this model of 30% undernutrition to investigate adipoinsular axis function. Maternal undernutrition followed by catch-up feeding through lactation reliably induced obesity and diabetes in offspring, with permanently altered plasma leptin levels and leptin receptor populations in the endocrine pancreas.

Vickers and his laboratory persisted with this model of severe food restriction – see for example the series of studies investigating the role of IGF-I, growth hormone and leptin on ameliorating the effects of developmental programming (122-124). Their rationale was that this regime of under-feeding reliably produced programming of obesity and the metabolic syndrome and produced a model with a phenotype akin to that of the human metabolic syndrome.

Various levels of undernutrition have been used by a range of laboratories, with a number of different measurement outcomes, typically associated with symptoms of the metabolic syndrome. Results have varied depending on the magnitude of the food restriction and the timing of its application. Sensitive periods of developmental plasticity are likely different for different organs, so timing of dietary manipulation during discreet periods of gestation will differentially target and shape anatomy and physiological function, with effects then persisting into adulthood.

B1.4 Rodent Models of "Overnutrition"

To provoke undernutrition in laboratory rats requires relatively straightforward dietary manipulation. The concept of 'overnutrition' however is more complex – how do you persuade a rat, which self-regulates for calories, to passively over-consume, to eat more than *ad libitum*? There are various ways of achieving true overnutrition in the rodent model, as outlined below. However, in our study, we stopped using the term 'overnutrition' because our model was not a true form of overnutrition; it was more correctly an *ad libitum* obesogenic high fat diet.

Artificial Intragastric Feeding This is a true model of overnutrition. The pup is separated from its mother at 4 days of age and a feeding tube inserted across the abdominal wall into its stomach so that quantifiable amounts of selected nutrition can be administered directly into the animal's digestive tract - Srinivasan *et al.* referred to this as their 'pup in a cup' model (125, 126). They used this preparation to investigate how diet during the postnatal period might alter or programme the phenotype - "lifetime growth trajectory was programmed by just 3 weeks of such a dietary intervention" p.16, (125). A feeding formula high in carbohydrates resulted in hyperinsulinemia, increased preproinsulin gene expression and adult-onset obesity. This model, however, is in some ways problematic given the known stressors associated with maternal separation and the effects of litter huddling on thermogenic responses and behaviours (127-129).

Reduction of Litter Size An average litter in rats is about 12 pups, with slight variations between common strains such as the Wistar and Sprague-Dawley. Reduction in litter size, for example down from 12 pups to only 3, leads to neonatal overfeeding due to the abundance of maternal milk supply. Such pups exhibited increased weaning weights with the accelerated weight gain continuing into adulthood (130). These rats were hypertensive, hyperinsulinemic and obese as adults, with an increased susceptibility to T2DM. There does not seem to be an accurate measure in this model of exactly how much milk and how many calories each rat pup consumed during lactation, either pups from treatment groups or controls. Instead, overnutrition was simply defined by any effects produced on phenotypic outcome, relative to Controls.

Maternal HF-feeding Although prenatal growth restriction has demonstrable influence on long term adiposity, it is important to recognise that the relationship between birth weight and later life pathophysiology is not linear. Moreover, although undernutrition remains a serious problem in many developing countries, worldwide there is an increasing focus on the rise in maternal nutritional excess and obesity as a risk factor in the health of offspring. Many studies have now modelled maternal nutritional excess using a number of different strategies, all

resulting in a rise in the incidence of adult obesity in offspring plus related metabolic disorders, although the timing and the magnitude of the phenotype varied according to the nutritional insult (131-133). A number of studies have now shown that programmed obesity may represent a U-shaped curve with a higher prevalence of adult obesity occurring in individuals who were on either low or high planes of maternal nutrition (77, 131, 134, 135).

Ghosh *et al*. (136) used a 20% fat diet for 10 days prior to mating and then throughout pregnancy and lactation – the resultant offspring were hypertensive. Taylor *et al*. (137) also used a 20% lard diet - these pups were growth retarded, and went on to develop cardiovascular dysfunction and hyperinsulinemia. (All the HF diets in this paragraph are percentage by weight – that is, a 20% fat diet contains 200g of fat per kilogram of diet.) A diet of 27.5% fat was used by Khan *et al.* (138) who found an increase in blood pressure among female but not male rats. Vascular dysfunction and plasma lipid irregularities were also produced by a 30% fat diet, utilised by Koukkou *et al.* (139). Guo & Jen applied a 40% HF diet and at weaning the offspring weighed more, had increased percentage body fat and elevated plasma glucose concentrations over controls (140). Work by Morris & Chen has also shown that established maternal obesity in the rat can reprogramme hypothalamic appetite regulators and leptin signalling at birth (134). In mice, it has been reported that a maternal HF diet can impair mammary gland development and result in lactational failure and high pup mortality (141).

High fat:high sugar diets and cafeteria diet approaches have also been utilised with a common phenotype of obesity and features of the metabolic syndrome (74, 142). The cafeteria-style diet is ‘highly palatable’ and ‘obesity-inducing’ (143), and is thought to more closely reflect a modern western-style diet among humans - much more so than, for example, the low protein diet. Indeed, the diet may consist of “supplemented chow, meat pies, pasta and cakes” p.385, (144), although most cafeteria-style diets have attempted to control the balance of fats, sugars and micronutrients more carefully than this. The high fat:high sugar cafeteria diet has been used to induce metabolic syndrome-type symptoms in IUGR offspring of undernourished dams (145, 146) - a model of catch-up growth. Or, it may be applied to dams earlier to induce obesity during pregnancy and *in utero* programming of offspring (134, 147).

It is also notable that many applications of maternal obesogenic diets in the rodent have resulted in either no change in offspring birth weights or reduced birth weights. Although maternal obesity in the human setting is often associated with large-for-gestational-age babies, this is usually manifest as a result of gestational diabetes, and it is now well established that maternal

obesity is also increasingly characterised by an increased susceptibility to IUGR/SGA babies, possibly due to placental insufficiency (148-150).

Note however, that it is unclear whether these studies produced results that were due to changes in gene expression/epigenetic modifications in the germline (the only true way of transgenerational transmission) or due to an abnormal intrauterine environment.

B1.5 Summary of rodent models of developmental programming

Both maternal undernutrition and maternal obesogenic diets produce developmental effects in offspring by mechanisms that are as yet largely unknown. Muhlhausler (151) focused on the appetite regulatory neural network within the hypothalamus as the central driver that was altered by perinatal nutritional influences, and programmed in a way that persists into adult life. A fully functioning food intake regulatory system is present at birth in most species – this would seem obviously necessary for survival. However, in the rat this neural circuitry is not fully mature until 16 days after birth (152), and thus the critical windows of developmental malleability are prolonged into the postnatal period.

Thus the critical periods for programming of appetite and growth and the metabolic syndrome in rodents are both pre- and postnatal. The postnatal period of lactation alone can be manipulated to induce programming, or to modify programming that has occurred earlier during gestation. Oscai & McGarr (153) stated, “the amount of food consumed during suckling in the rat plays an important role in determining subsequent food intake in later life.” The phenomenon of ‘catch-up’ growth is an example of the influence of postnatal nutrition on weight gain and on the risk of disease states in adult life. Because the period of developmental plasticity extends through both gestation and lactation in the rat, the current study was designed to explore the effects of dietary manipulation during either or both of these periods, utilising both maternal undernutrition and maternal HF dietary approaches.

B2 Developmental Programming of the Rodent Pancreas

Normal Development of the Rodent Pancreas

There is a rapid increase in the numbers of pancreatic β-cells during late gestation in the fetal rat (154). β-cells replicate and undifferentiated β-cell precursors are recruited and mature within the pancreatic ducts. This rapid proliferation declines within 3-4 days after birth, and the fetal islet cell mass is then subjected to a phase of remodelling and apoptosis at age 2-3 weeks, around the time of weaning. Thereafter β-cell production continues to slow until a stable state of cell turnover is reached, meaning that there is a relatively fixed mass of pancreatic islet cells in adult life. Normally in adult rats this mass of cells represents a considerable functional reserve.

The early 'fetal-type' β-cells produce insulin in acute response to amino acids, but are poorly responsive to blood-glucose levels. These cells are then lost and replaced in the phase of apoptosis and remodelling, with the second generation β-cells being much more acutely attuned to glucose levels in the way they secrete insulin. Hill & Duvillie state: "Because β-cell plasticity after the perinatal period is limited, a dysfunctional programming of β-cell ontogeny may present a long-term risk factor for glucose intolerance and type 2 diabetes" (155).

Prenatal pancreatic development

The developing fetal pancreas seems to be particularly sensitive to altered nutrient supply. In conditions of maternal undernutrition the fetus preferentially favoured some organs, in particular the brain, at the expense of visceral organs such as the pancreas and the liver (86). The result was morphological and functional changes in these visceral organs, which may account for the adult phenotype having an increased risk of developing diabetes.

Restricted nutrient supply led to fetal IUGR and this included reduced neogenesis of pancreatic beta cells (156). The total number of pancreatic cells was reduced, including β-cells and other islet cells, so that less insulin was detected, both within the pancreas and in the circulation. These structural and functional changes persisted into adulthood, as Garofano *et al.* (1997) state: "*in utero* undernutrition in rats … durably impairs β-cell development" p.1231, (157); it became an "irreversible developmental deficit" p.600, (158).

Interestingly, some interventions can rescue β-cells. Taurine supplementation in low-protein fed dams restored β–cell mass in offspring (159, 160). Similarly, administration of exendin-4, a pancreatic β-cell trophic factor, to neonates suffering IUGR due to uteroplacental insufficiency, prevented loss of β-cell mass and the subsequent development of T2DM (108). These findings

suggest that the deleterious effects of developmental programming are not always irreversible. Moreover, Vickers *et al*. (2005), although not measuring β-cell mass directly, demonstrated preservation of islet function in adult offspring in a model of maternal undernutrition, through administration of leptin during the postnatal period (123). And in a recent paper, maternal antioxidant supplementation during pregnancy limited the development of adiposity in offspring of rats fed an obesogenic diet (161).

Postnatal pancreatic development

Postnatal nutrition alone can also affect pancreatic development. This was seen when normal control rats were transitioned to a higher caloric plane during lactation through reduction of litter size (162), or by artificial intragastric feeding (125, 126). Such pups had raised numbers of islet cells and increased insulin secretion. This programming persisted into adulthood, producing full blown obesity by day P100. It also persisted into the next generation – when the F1 generation were mated, they gave birth to hyperinsulinemic offspring who also went on to become obese (125). [1]

Waterland & Garza (162) also investigated postnatal nutrition and its effect on pancreatic development. They varied litter size to create under- and over-nourished groups relative to Controls, and then tested the groups for insulin tolerance and glucose tolerance. Both the small- and large-litter groups demonstrated normal insulin tolerance (i.e., they did not show insulin resistance), but both groups showed a defective pancreatic insulin response to IV glucose challenge (a glucose tolerance test, or GTT). They deduced that the endocrine pancreas was the primary component of the insulin axis affected by such nutritional programming, rather than skeletal muscle – as they stated, "the endocrine pancreas is a repository of 'metabolic memory' in this model of metabolic imprinting" (p.358, (162)).

Transgenerational effects

It is noteworthy that the programming of endocrine function due to maternal undernutrition had transgenerational effects. When the hypoinsulinemic IUGR offspring of undernourished dams

[1] Interestingly, all the pups in the Srinivasan *et al.* experiments 125. Srinivasan M, Laychock SG, Hill DJ, Patel MS. Neonatal nutrition: metabolic programming of pancreatic islets and obesity. Experimental biology and medicine (Maywood, NJ. 2003;228(1):15-23, 126. Srinivasan M, Aalinkeel R, Song F, Mitrani P, Pandya JD, Strutt B, et al. Maternal hyperinsulinemia predisposes rat fetuses for hyperinsulinemia, and adult-onset obesity and maternal mild food restriction reverses this phenotype. American journal of physiology. 2006;290(1):E129-E34. received the same amount of calories, except that the treatment group were fed an isocaloric mixture rich in carbohydrates, in contrast to maternal rat milk where fat supplies the bulk of calories. An artificial feeding control group who were fed an intragastric formula equivalent to rat milk did not develop hyperinsulinaemia or adult onset obesity. (The composition of this formula, and of rat breast milk is: carbohydrate 8%, protein 24%, and fat 68%.) Because rat milk is high in fat, insulin secretion in suppressed in normal pups raised under normal conditions. The high-carbohydrate diet fed intragastrically immediately increased the demand for insulin and the pancreatic islets adapted accordingly; and because this response occurred during a critical window of developmental plasticity, pancreatic function was programmed, a durable effect persisting into adult life - indeed persisting transgenerationally.

were in their turn mated (with control fathers), the resultant fetuses (the F2 generation) were born with reduced β-cell mass, diminished insulin content and islet numbers – risk factors for that generation also developing diabetes (163). In a similar model, utilising a maternal low-protein diet, Burdge et al (2008) demonstrated that induced phenotypic changes persisting to the F2 generation were transmitted by epigenetic mechanisms (164).

Adipocytes

Fetal programming brought on by a maternal low-protein diet affected insulin sensitivity in some adipose tissues – for example, there were increased numbers of insulin receptors in epididymal and intra-abdominal adipocytes (165, 166). Similarly, macrosomic pups born to diabetic mothers developed obesity and hyperinsulinemia in adult life, and had adipocytes with an altered morphology and a lessened response to insulin (167, 168).

So it appears that the adipocyte and its role as part of the adipoinsular axis can be programmed by fetal and/or developmental malnutrition. This current study did not investigate the adipocyte directly, but we did quantify gross adiposity in our experimental animals, utilising DEXA technology. We observed dramatic changes in percent body fat in offspring induced by maternal diet. There is a powerful association between obesity and T2DM in humans (169), and I will focus on these two areas in the sections that follow.

Part C: Insulin, Leptin & the Adipoinsular Axis

C1 Insulin

The β-cells are the most common of the Islets of Langerhans cells in the pancreas, making up about 60-70% of the total islet mass. They are the only insulin producing cells in the body.

Insulin is a polypeptide consisting of two chains of amino acids (the A chain and the B chain) that are linked together by two disulphide bridges, with an intra-chain disulphide bridge found also within the A chain. There are 21 amino acids in the A chain, 30 in the B chain. Insulin is synthesised as part of a larger preprohormone. Preproinsulin is translated from the RNA in the nucleus, loses its leading signalling peptide when it enters the endoplasmic reticulum to be folded by the formation of the disulphide bridges, thus becoming proinsulin. Proinsulin becomes insulin when a segment of peptide between chains A and B is removed. The section of connecting peptide is termed the C-peptide, and equivalent amounts of C-peptide are released from insulin granules when insulin is secreted. Thus assaying the amount of circulating C-peptide is a convenient way of determining B-cell function, especially in patients who self-administer exogenous insulin (170).

There are minor variations in insulin amino acid sequence between mammalian species. Historically, animal insulins (primarily bovine and porcine) were used to treat diabetes in humans – indicating that the molecular differences are not sufficient to disturb biological activity. However, those receiving commercial animal insulins typically developed anti-insulin antibodies, and in some cases that affected the efficacy of the injection. Antigenicity was lowest with pork derived insulin which differs from the human in only one amino acid residue. Current commercial insulin is human-identical, produced in bacteria with recombinant DNA technology - primarily to avoid formation of such antibodies (170).

Rats have two genes coding for insulin (Ins1 and Ins2) and these secrete two different insulins. The differences are molecular rather than functional. This may reflect the physiological importance of the insulin molecule, that evolutionarily its function has been conserved.

Insulin is a hormone that powerfully regulates and stabilises plasma glucose levels. All its actions are anabolic and toward lowering of blood glucose concentrations. Islet β-cells function as sensors, monitoring plasma glucose levels via a copious blood supply. They are also effectors,

secreting insulin in quantities tailored to maintain plasma glucose levels within strict homeostatic bounds, with muscle and liver and adipose tissues as their main targets of insulin action.

C2 Glucose-stimulated insulin secretion

Glucose freely enters the β-cell through the GLUT2 transporter protein, diffusing down its concentration gradient from the plasma. Inside the cell it is immediately converted into glucose-6-phosphate (G6P) by the enzyme glucokinase B (GKB), which determines the rate of glucose-stimulated insulin secretion (GSIS). G6P is converted to pyruvate in the cytoplasm and then enters the mitochondria where it is metabolised via the citric acid cycle. The end products of this metabolism are carbon dioxide, water and ATP (adenosine-triphosphate, the energy unit of the cell). This, in turn, inhibits the ATP-sensitive potassium channel, reducing potassium efflux. Decreased movement of K^+ across the cell membrane leads to a depolarisation of the membrane which opens voltage-gated calcium channels and allows an influx of calcium. Increased intracellular Ca^{2+} stimulates the movement of insulin vesicles to the cell membrane and the exocytosis of their contents. Thus, by this chain of events, secretion of insulin is directly linked to circulating glucose concentrations. (See Figure 1.1)

Glucose also stimulates transcription of the preproinsulin gene, via the essential transcription factor Pdx1, which binds to the preproinsulin gene promoter site. The preproinsulin mRNA is then transported out of the nucleus and further modified to form the two-chain molecule, insulin (and in the case of rats, two insulin genes and two functionally identical insulins, Ins1 and Ins2).

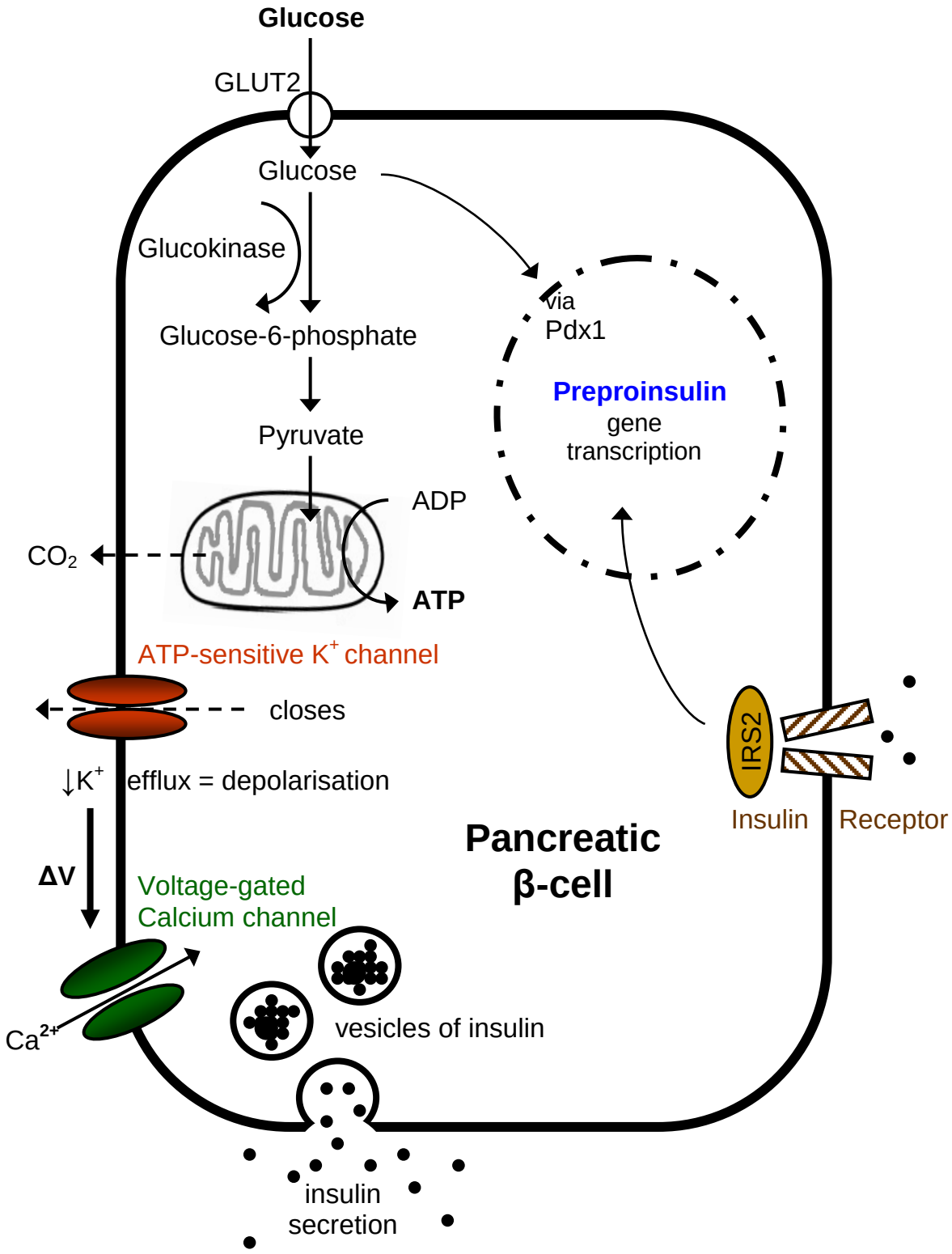


Figure 1.1: **Glucose-stimulated insulin secretion**

Glucose is taken up by the GLUT2 transporter, phosphorylated by glucokinase, leading to increased ATP production. ATP-sensitive K^+ channels close, provoking cell depolarisation and influx of Ca^{2+} ions, triggering exocytosis of insulin.

Glucose regulates preproinsulin expression via Pdx1 and other transcription factors.

Secreted insulin activates β-cell insulin receptors, phosphorylating insulin receptor substrate-2, recruitment of PI3K, and leading to preproinsulin gene transcription.

C3 Leptin

A satiety signalling molecule was hypothesised by Coleman in 1973 (171) following parabiosis experiments, partially fusing the circulations of *ob/ob* and *db/db* mutant mice with wild type animals. (*ob* stands for the 'obese' gene, and *db* for the 'diabetes' gene.) Obese (*ob/ob*) mice did not seem able to produce the circulating satiety factor themselves, but when fused to the circulation of a normal mouse, demonstrated a normal and appropriate response to it. By contrast, diabetes (*db/db*) mice appeared to manufacture the factor but were unable to respond to it. Coleman accurately hypothesised that the *ob* gene was responsible for a circulating satiety molecule and that the *db* gene encoded a receptor for this *ob* gene product. Furthermore, he proposed that the hypothalamus was involved, given that organ's known role in appetite regulation.

Leptin was discovered by Friedman and colleagues in 1994 (172), a 167-amino acid polypeptide weighing 16kDa, with a high homology across species - human leptin is 84% identical to mouse leptin and 83% identical to rat leptin (173). Leptin is primarily produced by the adipocyte, by white adipose tissue (WAT), at a concentration proportional to body fat stores (172, 174). Women have higher leptin levels than men of the same body mass, due to a higher proportion of WAT in the subcutaneous depot, and because leptin synthesis is stimulated by estrogens but inhibited by androgens (175, 176). (The opposite is true in rats – male rats have higher body fat content and higher leptin levels than females (177).)

Leptin has a major role in central regulation of appetite and food consumption via its interaction with the hypothalamus (178-180). Leptin acts as a lipostat, signalling the extent of body fat storage to the brain (174, 181). Weight gain and increased adiposity increases leptin secretion, which increases melanocyte-stimulating hormone in the arcuate, paraventricular nuclei (PVN) and lateral hypothalamus, with resultant decreased food intake and increased energy expenditure and sympathetic tone. Conversely, weight loss and decreased leptin secretion releases inhibition of neuropeptide Y (NPY) whose levels increase in the medial arcuate, leading to increased food intake and parasympathetic tone, along with decreased energy expenditure (182-184). Leptin appears to have a longer time-span modulating function rather than signalling acute changes, such as might occur after a single meal (185). A more chronic change in food intake, either an increase or decrease over several days, affects leptin secretion out of proportion to the corresponding change in fat mass (173, 174, 186). Leptin may therefore not be an anti-obesity hormone as such - the 'thrifty phenotype' hypothesis suggests that adapting to undernutrition has been a more common evolutionary pressure for most species, rather than excess and obesity (187, 188).

Moreover, obese individuals develop resistance to leptin, blunting the stimulus of hyperleptinemia. It seems therefore that leptin may primarily be a hormone involved in nutritional deprivation, to ensure survival in famine or settings of starvation, rather than preventing the development of obesity in times of plenty (189).

Leptin also influences numerous other tissues and physiological functions – reproduction, neuroendocrine signalling, and insulin action (190-194). Of particular interest to this thesis is its relationship with the pancreas, with the insulin-secreting β-cells in the Islets of Langerhans.

C4 Leptin Receptors

The leptin receptor was first reported by Tartaglia *et al* (1995) in the mouse (195). In 1997 the same group demonstrated that the *db/db* mutation was a defect in the leptin receptor (196), confirming Coleman's prediction of more than two decades earlier. Similar receptor mutations are observed (though extremely rarely) in humans (173), producing a similar pattern of symptoms - hyperphagia and early onset obesity (197).

The leptin receptor has a single membrane spanning region and a cytoplasmic tail; it is a member of the class I cytokine receptor family, similar to the interleukin 6-type (IL-6-type) cytokine receptors (196). Like other cytokine receptors, the leptin receptor depends on ligand-induced phosphylation of intracellular tyrosine kinases for signal transduction (198, 199).

There are thought to be at least 6 isoforms of the leptin receptor, termed ObRa through to ObRf (196, 200), alternative splicings of the same singular Lepr gene. These receptors have been characterised into three categories: a long form which is most physiologically active in signal transduction (ObRb); several isoforms with truncated intracellular domains (ObRa, ObRc, ObRd, ObRf), of which ObRa is thought to have some physiological activity and may be involved in transport of leptin across the blood-brain barrier; and ObRe which remains soluble because it lacks the membrane spanning region. Leptin receptors (in *rattus norvegicus*) typically have an extracellular region of 889 basepairs with conserved sites for ligand binding, plus a membrane-spanning region and an intracellular tail. Of these receptors 5 have identical transmembrane spanning regions, with ObRe alone lacking this motif – thus ObRe is not anchored within the cell membrane, but remains soluble within the circulating plasma. It is thought to have a role in binding the leptin hormone in the circulation and modulating bioavailability (since only free ligand is biologically available and active.)

ObRb is known as the long form of the leptin receptor – it has the longest intracellular tail. This intracellular region contains two sites of particular interest known as Box 1 and Box 2 which are binding sites for JAK and STAT respectively, which mediate the work of the ligand-receptor complex within the cell. ObRb alone contains both the Box 1 and 2 motifs and this receptor isoform appears to be the most physiologically active of all the variants. Mutation of the ObRb receptor, so that it lacks any functional activity is seen in the diabetic *db/db* mouse, and leads to obesity and diabetes (173).

Early papers studying the leptin receptor tended to refer to only two receptor isoforms (196), which were labelled either the long form (ObR_L) or the short form of the receptor (ObR_S), on the basis of the length of their intracellular tail. The long isoform was demonstrated to be physiologically active whereas the short form was thought to lack the intracellular apparatus required for intracellular signalling and was therefore often labelled as 'of unknown function'. Curiously the short isoform appears to be the more abundant (201, 202).

Uotani et al. (1999) showed that both receptor types are involved in uptake and degradation of leptin. Leptin is internalised into the cell by endocytosis of the receptor and ligand. The receptor and ligand separate within the endosome, the receptors recycle to the cell membrane while the leptin is transported to and fuses with a lysosome where degradation of the ligand takes place (203).

C5 Leptin Receptor Signalling

Leptin receptors are located on the cell membranes of pancreatic β-cells (191, 204) and leptin has direct inhibitory action on insulin secretion (205-208). The mechanisms by which leptin suppresses insulin secretion appear to be two-fold (209):

- It directly interferes with the glucose-stimulated insulin release pathway
- It alters gene expression (being the more 'indirect' mechanism)

Binding of the leptin receptor leads to activation of multiple intracellular signal transduction pathways (210). See Becker (2009) (211) for a review, on which the following outline is based.

C5.1 The JAK-STAT Pathway

The intracellular tail of ObRb, the 'long form' of the leptin receptor, contains sequences (the highly conserved so-called Box 1 and Box 2 motifs), to which cytoplasmic signalling molecules bind – JAK2, of the *Janus* kinase family (201). The leptin receptor is homodimeric, and when activated by ligand-receptor binding a conformational change is thought to mediate the autophosphorylation of at least two JAK2 molecules at the Box motifs. Activated JAK2 in turn then phosphorylates three conserved intracellular tyrosine residues located on the receptor's intracellular tail - Tyr^{985}, Tyr^{1077} and Tyr^{1138} (212). These 'phosphotyrosines' provide docking sites for various signal transduction proteins with specialized and specific binding domains – these are the Src homology 2 (SH2) domains. If the amino acid sequences are optimal, SH2 domains bind to the phosphotyrosines with high affinity (213). The best characterised of the JAK substrates is the family of proteins called signal transducers and activators of transcription (STATs) (214). STATs remain latent in the cytoplasm until they become activated through tyrosine phosphorylation, and on the ObRb the SH2-containing STAT3 is recruited specifically to Tyr^{1138}. The STAT3 molecules are phosphorylated by the receptor associated JAK kinases, and then dimerise to form homodimers. The phosphorylated STAT3 homodimer is then actively translocated to the nucleus. See Figure 1.2 below. Becker (2009) refers to phosphorylation of STAT3 as the 'most robust downstream effect of leptin receptor activation' (p.3, (211)).

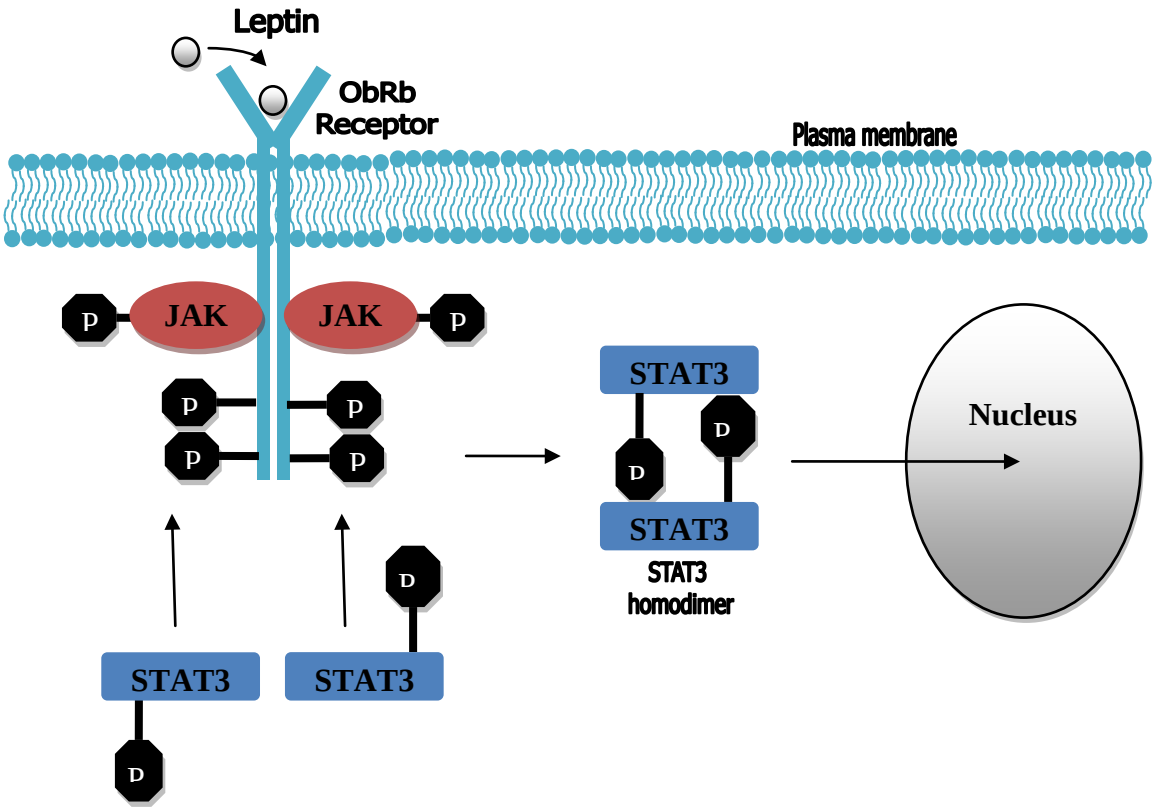


Figure 1.2: **The JAK-STAT signalling pathway**

Leptin binds to its pre-formed homodimeric receptor, ObRb - this results in auto-phosphorylation of associated JAK2. Activated JAK2s then phosphorylate the ObRb at target tyrosine sites such as Tyr^{985} and Tyr^{1138}. Activated Tyr^{1138} is a docking site specific for STAT3.

Once recruited to the Tyr^{1138} site, the STAT3 molecules are themselves tyrosine-phosphorylated by the JAKs. Two phosphorylated STAT3s dimerise to form a STAT3 homodimer, which is actively transported to the nucleus. (Modified from Williams, 2000)

Once inside the nucleus the active STAT3 homodimer binds to sequence-specific promoter regions and activates transcription of target genes. For example, STAT3 activates transcription of suppressor of cytokine signalling 3 (SOCS3) (215), one of the genes investigated in the current study. SOCS3 blocks the transcription of the preproinsulin gene (216) and causes a decrease in insulin receptor signalling (209). SOCS3 also negatively feeds back on leptin receptor activity – it inhibits ObRb recruitment of STATs by binding to the Tyr^{985} tyrosine residue site (215), and can disrupt JAK2 activity directly (217). Other target genes of leptin via the STAT3 pathway include increased transcription of the hypothalamic gene proopiomelanocortin (*Pomc*) (218), and the negative regulation of the genes for the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) (219).

Ligand-dependant STAT activation is relatively transient, lasting minutes to hours (214). Nuclear phosphotases de-phosphorylate the STAT protein, inactivating it and transporting it out of the nucleus (220).

C5.2 The IRS / PI3K / PIP_3 Signalling Pathway

Receptor-activated JAK2 is also involved in another signalling pathway, via the insulin receptor substrates (IRS1 & IRS2), through phosphoinositide 3-kinase (PI3K), and PIP3.

IRS-1 and IRS-2 are familiar as key proteins in the insulin receptor (IR) signalling pathway, where they are important regulators of insulin action and glucose homeostasis (221, 222). (Interestingly, in addition to being key to the insulin pathway, IRS can also be phosphorylated by a number of other ligands, including both the long and short forms of the leptin receptor (201, 223). However, activation of IRS/PI3K pathway in response to leptin is much less than that seen with insulin (211).) Once phosphorylated, IRS proteins become docking sites for SH2 domain-containing proteins, including phosphoinositide 3-kinase (PI3K). PI3Ks are heterodimers made up of a catalytic subunit (p110) and a regulatory subunit (p85) (224). The p85 subunit contains two SH2 domains, which link the p110 catalytic subunit of the PI3K to other phosphotyrosine-containing signalling proteins, such as phosphoinositide$_{3,4,5}$ triphosphate (PIP_3) (225).

PIP_3 binds directly to the ATP binding site of the pancreatic ATP-sensitive potassium channel, opening the channel and increasing K^+ efflux (226). Another way that leptin increases PIP_3 activity is by inhibiting PTEN (phosphotase and tensin homologue protein), which is a phosphotase enzyme that can dephosphorylate PIP3 (227). The end effect is enhanced potassium conductance, which hyperpolarises the cell membrane and reduces cell excitability, decreasing calcium influx via the voltage-gated calcium channel, leading to reduced insulin granule exocytosis. This is one of leptin's direct actions in suppressing insulin secretion (228). The ATP-sensitive K^+ channel is also the target of the T2DM sulphonylurea drug tolbutamide, which stimulates insulin secretion by inhibiting channel opening (229).

PIP_3 has other downstream effects. It stimulates protein kinase B (PKB, also known as Akt), which in turn activates phosphodiesterase 3B (PDE3B). PDE3B is an enzyme that reduces intracellular cAMP levels, (see Figure 1.3). It thus antagonises the action of glucagon-like peptide-1 (GLP-1), which is mediated by cAMP. Seufert (2004) stated that "the inhibitory actions of leptin on insulin secretion are primarily mediated through PI3K-dependent activation of PDE3B and a subsequent reduction of intracellular cAMP" (p.S155, (209)).

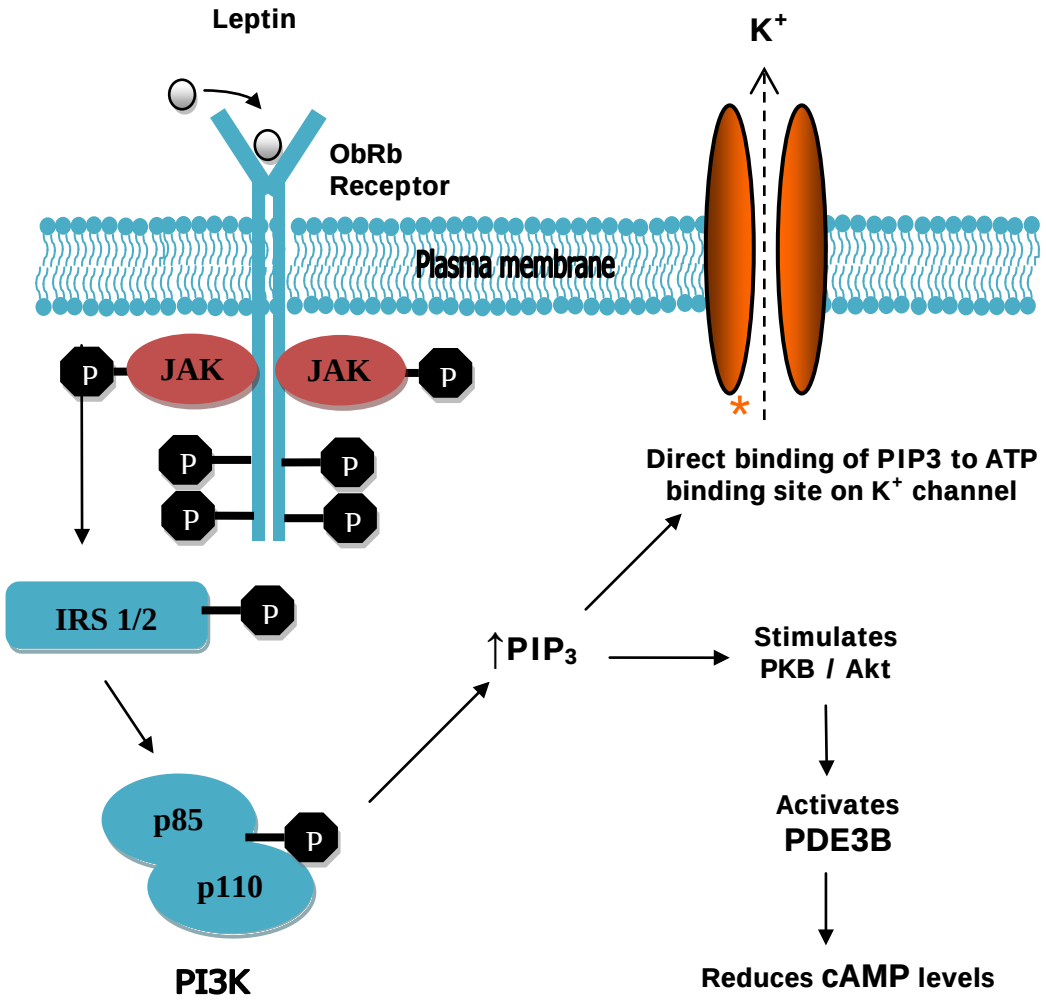


Figure 1.3: **The PI3K signalling pathway**

PI3K is a heterodimer made up of two subunits, p110 and p85 (230). Leptin binding activates PI3K via JAK phosphorylation of IRSs, and thereby increases the levels of PIP_3. Downstream of PI3K, PIP_3 reduces K^+ channel activity by two mechanisms: PIP_3 occupies the ATP binding site on the channel and increases K^+ efflux, and it reduces cAMP levels by activation of the enzyme PDE3B. (Modified from Alessi *et al.*, 1997)

C5.3 Other Signalling Pathways

Leptin and ObRb are also able to activate other signalling pathways in the β-cell. There are other molecules within the STAT family – STATS 1, 5 and 6 – which leptin can activate, at least in hypothalamic cell populations (231). Leptin also activates the ERK pathway which leads to regulation of gene expression; again, this has been demonstrated in hypothalamic neuronal cell lines (232), but the application to the β-cell is less known. Activation of the mammalian target of Rapamycin (mTOR) pathway, and the inhibition of the AMP-activated protein kinase (AMPK) pathway (in the hypothalamus) remain "poorly understood" (p.640, (233)).

In the current study we have investigated levels of mRNA expression of key candidate genes involved in the insulin secretion process, and in leptin's regulation of that process via the various pathways described above. These gene expression studies are reported in Chapter 5.

C6 The Adipoinsular Axis

Kieffer & Habener (2000) proposed a hormonal feedback loop between the pancreatic β-cell and white adipose tissue (WAT), and coined the term 'the adipoinsular axis' (234). In this axis, insulin secreted from β-cells stimulates the adipocyte to promote adipogenesis and leptin production, while circulating leptin, in turn, restrains the production of insulin from the β-cell. Insulin is adipogenic, that is, it promotes the uptake and storage of fat, and leptin secretion is directly proportional to fat mass (235). As adiposity increases and plasma leptin levels rise, insulin release is restrained and this places a limit on adipogenesis – an "endocrine brake to curtail further accumulation of fat" p.292, (236). In the opposite setting, if fat mass diminishes, there is less leptin released, and thus less restraint on insulin production and additional fat deposition takes place. This feedback axis is thus likely part of the homeostatic mechanism defending a set-point for body weight (237).

Although leptin and insulin have powerful central effects on appetite centres in the hypothalamus, the main concerns of this thesis are the effects of leptin on insulin secretion from the pancreatic β-cell. Kieffer & Habener (2000) produced the diagram reproduced below (Figure 1.4) to illustrate their concept of an adipoinsular axis (234). The axis functions to maintain nutrient balance, but dysregulation of the axis may contribute to obesity and the development of hyperinsulinemia associated with diabetes (208, 234). Where leptin levels are chronically elevated because of increased adiposity, leptin resistance develops: the β-cell becomes unresponsive to the leptin signal. Leptin restraint on insulin secretion is lost, the adipoinsular axis dysfunctions and hyperinsulinemia results. Furthermore, hyperinsulinemia exacerbates obesity by increased adipogenesis, causing increased leptin production. This becomes a positive feedback loop with leptin desensitisation in both the hypothalamus and the pancreatic β-cell resulting in hyperphagia and hyperinsulinemia, contributing to the pathogenesis of obesity-associated diabetes (238).

One crude proxy of adipoinsular axis function is the insulin:leptin ratio (235), where the relationship between concentrations of plasma insulin and leptin is quantified. Quantification of gene expression, by measuring relative mRNA levels of key axial genes, is another way of exploring adipoinsular functionality between treatment groups.

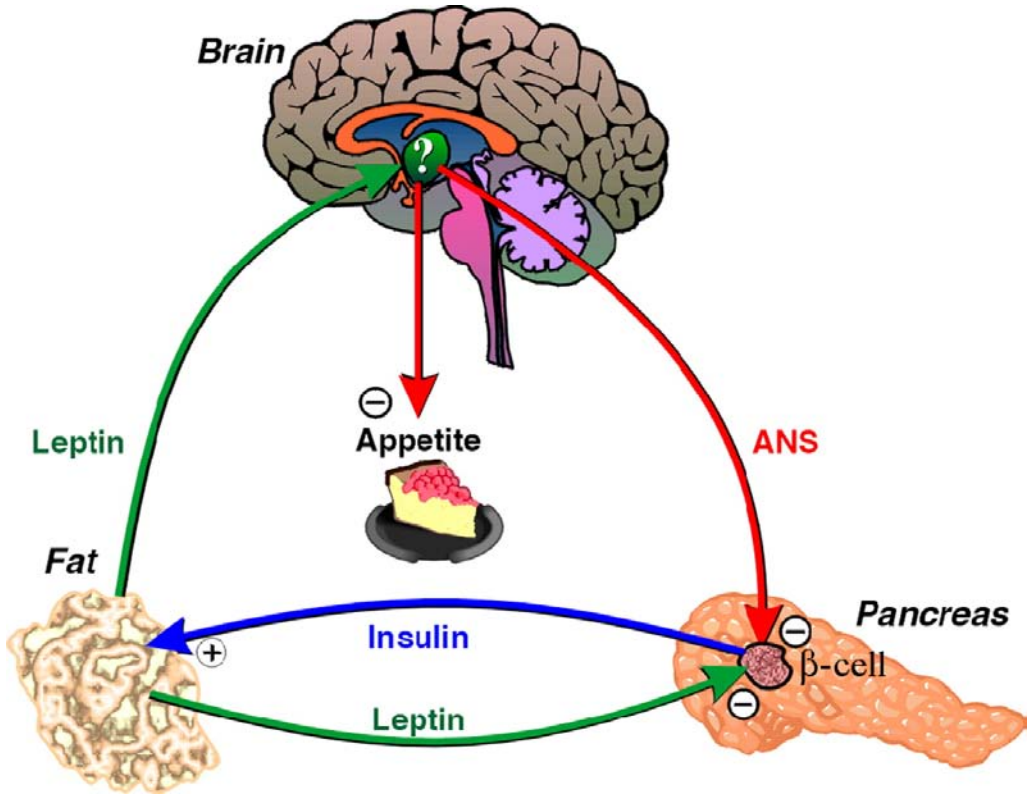


Figure 1.4: **The Adipoinsular Axis**

Insulin is adipogenic and increases the production of leptin by adipose tissue. Leptin feeds back to reduce both insulin secretion and insulin gene expression. The suppressive effect of leptin on insulin production is mediated both by the autonomic nervous system (ANS) and by direct actions via leptin receptors on β-cells. (Reproduced from Kieffer & Habener, 2000).

Part D: Obesity & Type 2 diabetes

D1 Insulin resistance and obesity

The β-cell is sensitive to plasma glucose concentrations, and plasma glucose sensing regulates insulin synthesis and secretion with great precision. In the absence of disease, plasma glucose and insulin levels parallel each other very consistently; it is the breakdown of this precise paralleling that constitutes the disease known as diabetes mellitus. In diabetes mellitus the essential action of insulin fails – either the body fails to produce enough, or its utilisation of insulin is faulty. The result is disordered blood glucose regulation. In untreated diabetics blood glucose levels rise: they become hyperglycemic. Hyperglycemia has a range of deleterious consequences, and in its most severe and untreated form is fatal.

Insulin resistance on the part of the insulin-responsive tissues (liver, muscle, fat) leads to hyperglycemia and hyperinsulinemia. Decreased glucose uptake by these tissues results in circulating hyperglycemia. Fatty acid levels also increase as stored triglycerides in adipocytes are broken down under the influence of glucagon. Insulin resistance by the liver leads to a derangement of the role of that organ in glucose homeostasis – uncoupled from the normal restraint of insulin, the liver continues to convert glycogen to glucose; similarly hepatic gluconeogenesis driven by glucagon continues unabated. The loss of control over these two hepatic functions because of insulin resistance is the major source of hyperglycemia in T2DM.

Muscle is the tissue where most glucose is utilised – "the major site of postprandial glucose disposal" p.606, (158, 239). Skeletal muscle develops both pre- and postnatally in the rat and is susceptible to alterations in maternal nutrition (239, 240). A downregulation of insulin receptor concentrations in skeletal muscle was seen in an undernutrition model (241), whereas altered gene expression of insulin-signalling proteins was evident in offspring of dams fed an obesogenic diet prior to mating (242).

Obesity is a major risk factor in the development of insulin resistance, with obesity found in over half of T2DM patients (243). It is central obesity that correlates with insulin resistance – this is the obesity of adipose tissue surrounding the inner abdominal visceral organs, as opposed to subcutaneous obesity where the adiposity is located under the skin. Abdominal fat is particularly hormonally active, secreting adipokines which induce insulin resistance. Leptin normally restrains insulin secretion, working directly via leptin receptors on the beta islet cells. However,

leptin resistance at the level of the pancreas undoes the restraint on insulin production resulting in hyperinsulinemia which in turn leads to insulin resistance in peripheral tissues (244).

Obesity not only produces hyperinsulinemia and dyslipidemia (high circulating fatty acids and triglycerides), but these, along with high blood pressure, damage blood vessels (atherosclerosis) and predispose to heart disease and stroke. This very commonly associated collection of symptoms lead Reaven in 1988 (245, 246) to formulate a condition he termed 'Syndrome X', now usually known as the Metabolic Syndrome. There are several definitions of what actually constitutes a diagnosis of Metabolic Syndrome – the details of the WHO definition differ somewhat from the American ATPIII (Adult Treatment Panel III), which in turn differ in emphasis from the International Diabetes Federation, but the essential components of the syndrome are the same – obesity, hypertension, dyslipidemia, hyperinsulinemia, impaired glucose tolerance (or worse forms of glucose dysregulation, i.e., full glucose intolerance or frank diabetes) (247).

The first approach to remedying insulin resistance and the onset of T2DM is to tackle obesity through physical exercise, diet (reducing carbohydrate intake) and weight loss (especially reduction of central adiposity.) These measures alone may well reduce insulin resistance, may restore insulin sensitivity to normal levels.

Obesity commonly leads to insulin resistance (245) - the reduced efficacy of insulin action. Indeed, obesity is the commonest cause of insulin resistance (169). However, most obese individuals, even though insulin resistant, don't go on to develop T2DM – i.e., they remain euglycemic. This is because their pancreatic β-cells rise to the challenge of insulin resistance by increasing insulin secretion (248). A diagnosis of T2DM is reached when the β-cell becomes unable to compensate for insulin resistance, at which point a loss of glycemic control develops (249).

β-cells can compensate to a remarkable degree. They respond to the challenge of insulin resistance by a variety of mechanisms:

increase in size of the β-cell (hypertrophy) (250);

increase in the amount of insulin released per β-cell (251);

self-replication of pre-existing β-cells (252);

neogenesis of β-cells from progenitor/stem cells. This certainly occurs in rodents – in a diabetic model (253), and during pregnancy (254). It has also been observed in humans in vitro

(255), but it is unclear what contribution such neogenesis makes to the increase in islet mass seen in insulin resistance, which is probably due more to β-cell hypertrophy.

The considerable compensatory capacity of the β-cell may be because there are some common human life-span events where an increase in insulin resistance is normal – for example: in puberty (256), in pregnancy (257), and in aging (258). Insulin secretion may increase 4-5 fold during these periods of natural insulin resistance (248, 256, 257, 259, 260). β-cell mass also increases, though less dramatically – by about 50% (250, 261). Thus the β-cell is shown to have remarkable functional reserves enabling it to compensate for insulin resistance – not only in natural settings such as puberty or pregnancy, but also in many cases of obesity.

D2 Insulin resistance and the development of T2DM

Insulin resistance precedes the development of T2DM. Insulin resistance is the inability of tissues to utilise insulin normally – such a tissue is said to exhibit an altered insulin sensitivity. Because insulin is unable to exert its normal effect on glucose transport across cell membranes, serum glucose levels remain elevated. Obesity is a prime cause of insulin resistance and there are several interconnected routes leading from excess adiposity to insulin resistance and T2DM. Two of these main mechanisms are: an increased release of adipokines – substances secreted by adipose tissue, and an elevated concentration of free fatty acids (FFAs) in the bloodstream. These two interrelated mechanisms will be briefly introduced here, then discussed more fully in the following sections.

Adipose tissue is most obviously a storage depot for ingested energy that is surplus to expenditure, but it is not an inert tissue – secreted adipokines have a dynamic role in the regulation of metabolism. A survey of gene expression in WAT revealed that 20% of active genes encoded for secretory proteins (262). Adipose tissue releases a number of adipokines that modulate metabolism: hormones such as leptin and adiponectin, proinflammatory cytokines, and FFAs (also known as non-esterified fatty acids or NEFAs) and glycerol.

Obesity raises plasma FFA levels – not only in those who go on to develop T2DM, but also in those who do not - and raised FFA levels are associated with the increased insulin resistance seen in both groups (263, 264). Kahn, Hull & Utzschneider (2006) describe FFA release as the ‘single most crucial’ element influencing tissue resistance to insulin action (265). Chronically raised levels of FFAs also impair β-cell function, interfering with insulin synthesis and normal insulin release in response to glucose (266). Thus elevated FFA levels appear to make a double

contribution to the development of T2DM – they make tissues more resistant to insulin action, plus they hinder the normal compensatory response of the β-cell. This provokes a feed forward momentum and a progressive ongoing decline in insulin functionality, seen in those obese individuals who go on to develop T2DM.

D3 Subcutaneous *versus* visceral fat deposition

The bodily location of the adipose tissue makes a difference to the development of insulin resistance and T2DM (267, 268). Visceral fat within the intra-abdominal depots is metabolically different to fat stored subcutaneously, and visceral adiposity makes a greater contribution to insulin resistance. This helps explain the heterogeneity of risk for T2DM seen in persons of similar BMI – those whose adiposity is located more centrally tend to be less glucose tolerant and more insulin resistant than the peripherally obese.

Visceral obesity occurs prior to the development of T2DM, in much the same way as do hyperinsulinemia and insulin resistance (269). Visceral adiposity is associated with an accumulation of fat in the liver, known as hepatic steatosis or non-alcoholic fatty liver disease (NAFLD). This leads to an excessive release of free fatty acids (FFAs) into the circulation. The proximity of the visceral fat depots to the liver (draining directly via the portal circulation) and the distinctive secretory profile of visceral adipose tissue (containing a higher proportion of proinflammatory cytokines) – these have a powerful effect on hepatic FFA levels, much more than does an equivalent peripheral fat mass.

D4 Insulin resistance and T2DM

The β-cell responds to insulin resistance and raised plasma glucose levels by compensatory increased insulin secretion. In most obese subjects the functional reserve capacity of the β-cell is sufficient to meet the increased demand for insulin. In others however (who perhaps have an underlying susceptibility), the β-cell dysfunctions, eventually leading to the development of T2DM.

By the time glycemic control is lost and a diagnosis of T2DM is reached, the number of β-cells has fallen by about 50% (250), and insulin secretion has dropped to about 25% of normal (270). It is apparent that insulin granules are still present inside the β-cells but they are not released as normal, either in response to glucose or to the stimulation of non-glucose secretagogues (249). This decline in β-cell numbers and function can be ongoing, which underlies the progressive nature of T2DM. In these individuals insulin secretion has become inadequate to meet demand and blood glucose levels climb and remain high, not only following food intake but even in the fasting state. The liver further exacerbates this state of hyperglycemia because the restraint exerted by insulin on hepatic production of glucose is lost. A constantly elevated blood glucose level may itself further damage β-cells, an effect termed glucotoxicity. The double burden of hyperglycemia plus elevated FFA levels potentiates damage to β-cells, and this has been referred to as glucolipotoxicty (271).

Insulin resistance develops and β-cell function declines long before glycemic control is lost. Obesity and the proinflammatory adipocytokines released by adipose tissue are the major contributors to this process, and the major determinant of obesity is energy imbalance – excessive nutrient consumption along with lack of physical exercise. Diet composition is important: long-term HF feeding has been shown not only to produce obesity but also to reduce insulin secretion in dogs (272).

D5 An underlying susceptibility in some people?

Not all obese people go on to develop insulin resistance or T2DM. They are sometimes referred to as ‘Metabolically Normal Obese People’. It is not known why some obese people are more vulnerable than others. It is thought that perhaps there is a genetic susceptibility in these people, that perhaps their genetic makeup is less protective. Another possibility lies in their developmental programming – in their prenatal and early postnatal nutritional experience - which

helps determine physiologic and metabolic parameters that persist into adulthood and which impact on adult disease susceptibility. For example, rats whose mothers were raised on a high fat diet during pregnancy and lactation were programmed to develop adiposity despite a normal postnatal diet (273).

Those who go on to develop T2DM may have an underlying susceptibility or vulnerability, either genetic or epigenetic, which propels them beyond the stage of compensation and into progressive β-cell dysfunction, inadequate insulin release and hyperglycemia. The single most important therapeutic action that an obese individual can take is to reduce weight, and the sooner this is done, the more β-cell loss of function can be avoided. Exercise and weight reduction are much more effective – nearly two fold - than a pharmacological intervention alone in reducing the progression from insulin resistance to T2DM (274).

Scope of this thesis

During pregnancy the developing fetus is dependent on its mother for all nutritional requirements. It is not surprising, therefore, that variations in maternal nutrition can be reflected in alterations in fetal health and well-being. Interestingly, evidence from epidemiological, clinical and experimental studies suggests that changes in maternal nutrition affecting the fetus can then persist into the offspring's adulthood, and may result in diseases such as diabetes, obesity and cardiovascular disease – the so-called 'lifestyle' diseases. The first observations of these phenomena led to the formulation of the Developmental Origins of Health and Disease (DOHaD) hypothesis. The first objective of this thesis is:

> *To test the hypothesis that maternal nutrition affects the life-long phenotype of the offspring in terms of the development of obesity and changes in insulin sensitivity.*

To this end we established two broad experiments within this animal study. Firstly, a cohort that examined the developmental programming effects of maternal undernutrition through the different developmental windows of pregnancy and/or lactation. (This is reported in Chapter 3.) And secondly, a nutritional manipulation utilising a maternal obesogenic diet during pregnancy and lactation, with or without pre-conceptional HF (Chapter 4). Programming and nutritional mismatch in offspring were further examined by utilised two levels of post-weaning diet: control Chow or a HF diet. Importantly all studies were designed to examine sex-specific programming effects, a feature missing from many reported studies where one sex was selectively used over the other and important sex effects were not investigated.

A reciprocal feedback relationship has been proposed between the two hormones leptin and insulin (234). This is the so-called adipoinsular axis, whereby insulin stimulates the release of leptin from white adipose tissue and leptin in turn restrains the insulin secretion from β-cells in the endocrine pancreas. Leptin has a central role in regulation of appetite (275, 276), is involved in the developmental tuning of central appetite mechanisms (277), and peripheral roles including the modulation of insulin secretion (208). A second objective of this thesis is:

> *To test the hypothesis that developmental programming will alter adipoinsular axial balance, and to investigate at a molecular level the mechanism/s of leptin restraint on β-cell secretion of insulin.*

The action of leptin on β-cell activity was investigated through relative gene expression studies of key genes involved in the glucose stimulated insulin secretion, plus the pathways downstream of

the leptin receptor that interact with insulin release, in the male offspring of the two maternal obesogenic dietary groups (Chapter 5). We hypothesised:

That because the phenotypes of the offspring of the two maternal obesogenic dietary groups were similar – both sets of male offspring exhibited increased adiposity, hyperinsulinemia and hyperleptinemia - that mRNA levels of key pancreatic genes would also be similar.

Obesity and its related metabolic disorders may prove to be the greatest threat to human lifestyle and health in the developed world this century. Earliest DOHaD studies focussed on the developmental threats of undernutrition during gestation and demonstrated that fetal growth restriction correlates with adult disease, implying that fetal nutritional deprivation is a strong stimulus for programming. Thus, experimental animal models were developed using controlled maternal caloric intake or protein or macronutrient deficiency. However, in many developed societies, maternal and postnatal caloric intake is either sufficient or excessive. Interestingly, both forms of nutritional insult, maternal undernutrition and maternal HF-consumption, can lead to obesity in offspring suggesting a "U"-shaped relationship between maternal nutrition and metabolic compromise in offspring. A third objective of this thesis is:

To establish possible commonality of mechanisms underlying developmental programming by using parallel models of both maternal undernutrition and maternal high-fat feeding.

There has been a dramatic decline in the age of menache in the Western hemisphere (278, 279). Moreover, there is a link between childhood growth and age of onset of puberty (280), and leptin has been shown to have a role in reproductive maturation (281, 282). (See both Chapters 3 and 4). Our experimental design enabled us to investigate a fourth hypothesis:

That maternal nutrition and/or nutrition during early postnatal life will impact on pubertal onset in offspring.

The final chapter evaluates the findings of this project and explores whether they lend support to the predictive adaptive responses (PARs) hypothesis (283).

Chapter 2: Materials & Methods

2.1 Introduction

2.1.1 Animal Experimentation

This study utilises a small animal model to examine the effects of altered maternal nutrition on the long-term development and health of the offspring. The rat has been used extensively in DOHaD research and the laboratory of Dr Vickers has over 20 years experience in nutritional modelling in the Wistar rat. All animal experiments were undertaken under the guidelines and approval of the Animal Ethics Committee at the University of Auckland. All animal experimental designs were performed with the best principles of laboratory animal experimentation in mind, that is the 'Three Rs' of Russell and Burch – Replacement, Reduction and Refinement, first proposed in 1959 (284-286), while maintaining the necessary statistical power as determined from prior independent experimental cohorts.

Replacement We felt that an animal model was required and could not be replaced by any other model for this study, which is informed in part by previous epidemiological observations in human populations, and which seeks to tease apart mechanistic determinants of the effects noted in humans. The Liggins Institute has developed animal models displaying a phenotype that closely resembles that of the human metabolic syndrome. The value of models utilising laboratory animals lies in the ability to manipulate one variable at a time whilst controlling against confounds, thus allowing for an examination of the interaction between the maternal and postnatal nutritional environments.

Reduction All endeavours were made to reduce the numbers of animals used, and to reduce ethical costs. Numbers were based on power equations derived from prior experimental cohorts in our laboratory, utilising the Wistar strain of rats. All work was done under the guidelines of the New Zealand Animal Welfare Act (287-289). Animals had free access to water at all times, and the level of undernutrition in the UN cohorts was at a moderate level. All other animals had food supplied *ad libitum*. Due to the social nature of rats, all animals were caged as pairs. The temperature and humidity of the housing facility were set at optimal levels and carefully monitored. A registered veterinary surgeon was available at all times should any animal appear unwell.

Refinement This refers to techniques of care, in all aspects, that minimise suffering and distress. Refinement comes with experience and with attention to animal welfare. The laboratory of Dr Vickers has extensive experience in large animal cohorts and as such has implemented a monitoring system that has been refined over numerous experimental cohorts to minimise disturbances to the animals, extraneous stressors and minimisation of handling stress.

All animal experimentation was approved by the University of Auckland Animal Ethics Committee (Application No. R402).

2.1.2 Broad Outline of the Experimental Design

Parental Generation Normal SPF-derived males and female Wistars were raised for breeding. They were placed on various diets during rearing as dictated by experimental protocols detailed below. Animals were acquired at a weaning age (day 22) to allow familiarisation with the handlers prior to mating.

Mating, Pregnancy, Lactation Dietary manipulation occurred during pre-conception, pregnancy and/or lactation. This study encompassed two broad categories of nutritional manipulation that resulted in a total of 6 maternal nutritional groups: Controls, 3 levels of undernutrition, and 2 levels of maternal obesogenic diets.

Offspring Litter size was adjusted at birth to 8 pups (4 male and 4 female) to ensure standardised nutrition until weaning. Weight gain and food consumption were monitored through lactation and during the postnatal period. At weaning the offspring were divided into male and female cohorts and further divided into 2 post-weaning dietary groups to be raised either on standard chow or HF.

Phenotypic measurements Body weights were recorded every three days during the post-weaning period and food intakes recorded at defined postnatal periods representing pre-pubertal, pubertal, mature adulthood and later adulthood. At day 150, body composition was quantified via dual energy x-ray absorptiometry (DEXA). At day 175, animals were killed (decapitation following anaesthesia) and plasma and tissues collected for later analysis. Plasma samples were analysed for leptin, insulin and glucose. Pancreas tissue was immediately dissected and snap frozen for gene expression analysis via qPCR.

2.2 Generation of the experimental cohorts

2.2.1 Animals for Breeding

Wistar rats were obtained from the SPF breeding colony at the Vernon Jansen Unit, the animal laboratory facility of the University of Auckland. As described earlier, animals were acquired at weaning age (day 22) to allow familiarisation with the handlers prior to mating and to provide a continuum of experimental handling and conditions throughout the course of the experiments. All breeders were housed under standard conditions with a 12:12 light:dark cycle, constant temperature and humidity and *ad libitum* access to food and water.

For the undernutrition cohorts, all females were fed a standard rat Chow *ad libitum* (Diet 2018, Harlan-Teklad, please refer to 2.2.2) from weaning until the time of mating. For the maternal high fat (HF) model, females were either fed the standard rat Chow *ad libitum* until mating or fed a moderate high fat diet (D12451, research Diets, please refer to 2.2.2) *ad libitum* from weaning until mating. A total of 76 females were used for breeding (56 maintained on the Chow diet and a further 20 fed the HF diet post-weaning).

Males for breeding purposes (n=20) were fed the standard rat Chow from weaning until the time of mating and housed under identical conditions to that of the females. Male background was normalised as much as possible across the maternal nutritional groups to minimise paternal/litter influences on experimental outcome.

All animal work was approved by the animal ethics committee at the University of Auckland.

2.2.2 Diet Composition

An outline of the two diets is given in Tables 2.1 and 2.2, and detailed compositional analysis is available via:

Chow diet, D2018: www.harlan.com

HF diet, D12451: www.researchdiets.com/pdf/Data%20Sheets/D12451.pdf

The HF diet utilised has been well cited in the literature and reflects an "open source" diet, thus ensuring consistency across batches. The standard control chow diet was routinely tested for compositional deviations and no significant differences were observed across batches during the course of these studies.

Table 2.1: **Composition of the standard control Chow diet (Harlan Teklad Diet 2018)**

Harlan Teklad Global Diet 2018	
Calories from protein	24%
Calories from fat	18%
Calories from carbohydrate	52%
Energy Density (kcal/g)	***3.1***
Macronutrients:	%
Protein	18.6
Fat (ether extract)	6.2
Carbohydrate (available)	44.2
Crude fibre	3.5
Neutral detergent fibre	14.7
Ash	5.3

Table 2.2: **Composition of the high fat (HF) diet (Research Diets D12451)**

Research Diets D12451	
Calories from protein	20%
Calories from fat	45%
Calories from carbohydrate	35%
Energy Density (kcal/g)	***4.73***
Macronutrients:	%
Protein	24
Fat	24
Carbohydrate	41

2.2.3 Growth & Weight Gain of Breeders

Animals of the parental generation were weighed twice weekly from weaning until the time of mating (day 100). Females were DEXA scanned prior to mating to examine the effect of pre-conceptional HF nutrition on body composition.

2.2.4 Mating

Mating commenced when the animals had reached 100 days of age. Females were probed using an EC40 Estrus Cycle Monitor (FST 22500-1 Rat, Fine Science Tools Inc., Canada) to detect the day of oestrus, at which time they were placed into a cage overnight with a selected male. Mating was confirmed the next morning by microscopic examination of material obtained by vaginal lavage. Presence of sperm and a vaginal mucus plug were indicative of successful mating. There were a few females where oestrus proved difficult to determine, even after a week of daily probing, and these were placed in cages with a selected male and left together for seven days to cover 1 oestrus cycle.

Female age range at mating was 103 – 119 days old, with the median at day 108.

Pregnant females were housed as singletons. Food intake and body weight were measured daily. Of the 56 females raised on chow, 44 were successfully mated (78.6% success rate). Of note, of the 20 females raised on the HF diet, only 8 were successfully mated (40% success rate). Following a two week mating period males and any females that did not become pregnant were euthanased, with tissues and plasma collected for pools.

2.2.4 Gestation

Once pregnancy was successfully achieved the females, with the exception of the pre-conceptional HF group, were randomly assigned to one of six different treatment groups spanning the 2 nutritional models. The undernutrition cohort required 4 groups: a control group and 3 levels of maternal undernutrition. The maternal obesity model required 3 groups: a control group and 2 maternal HF groups. These experimental groupings are outlined in Figures 2.1 and 2.2. Maternal body weights and food intakes were recorded daily throughout pregnancy. In particular, in the UN model, daily food intakes were a necessity so as to apportion 50% of Control intake to the undernourished dams. Length of gestation was recorded for all animals.

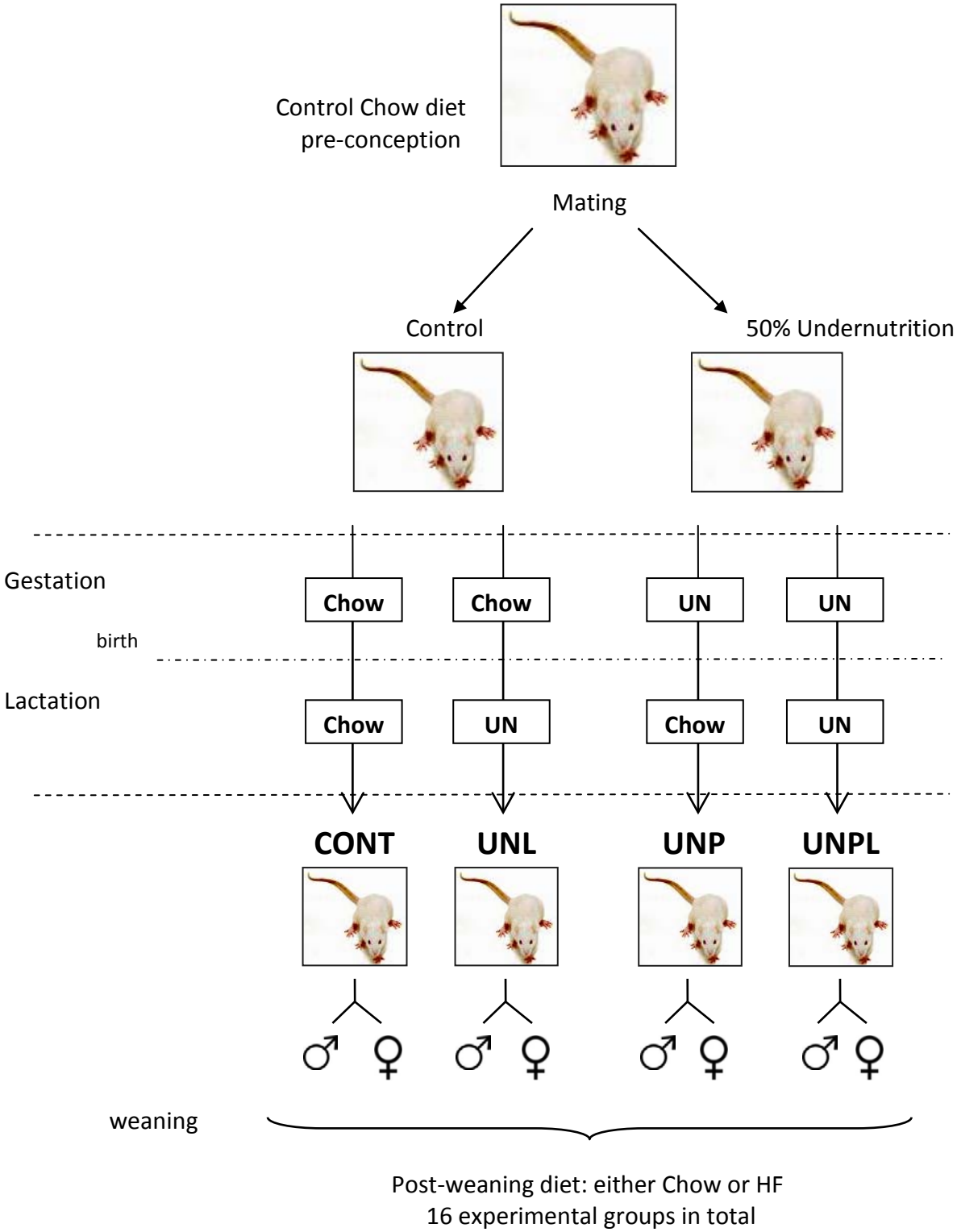


Figure 2.1: **Experimental model for maternal undernutrition study**

In a full balanced experimental design, a total of 16 (4x2x2) experimental groups were established encompassing 4 maternal nutritional groups, male *versus* female, and 2 levels of postnatal nutrition.

Ad-lib = *ad libitum* Chow diet; UN = 50% undernutrition diet (= 50% of Control consumption by weight); CONT = Controls, fed standard Chow; UNL = dams fed 50%UN during lactation only; UNP = dams fed 50%UN during pregnancy only; UNPL = dams fed 50%UN during both pregnancy and lactation.

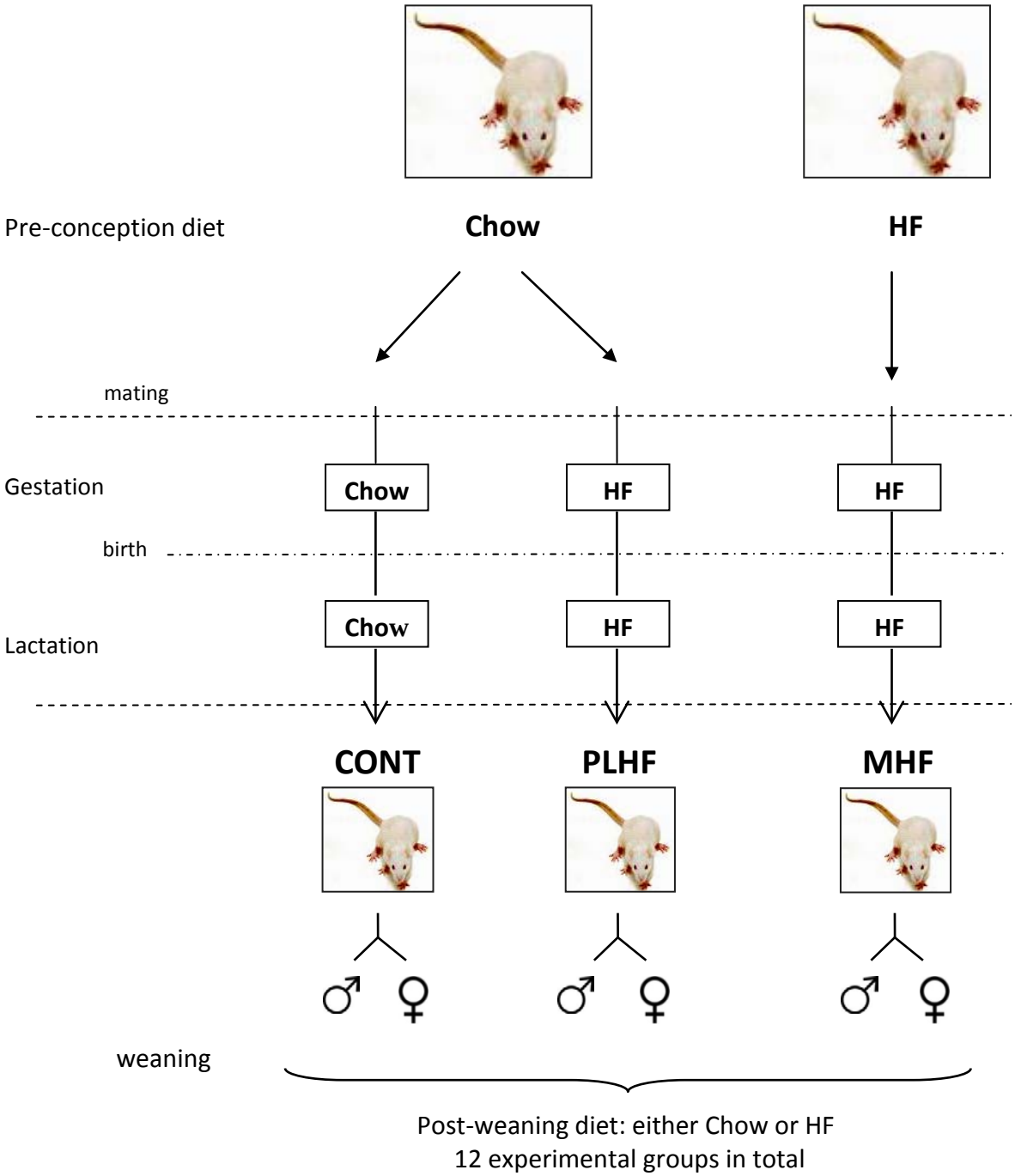


Figure 2.2: **Experimental model for maternal high fat study**

This experimental design resulted in a total of 12 (3x2x2) groups encompassing 3 levels of maternal nutrition, males *versus* females, and 2 levels of postnatal nutrition.

Note that a "reversibility" group (pre-conceptional HF diet then Control diet throughout pregnancy and lactation) was not incorporated as this was not part of the underlying hypothesis but will be examined in future independent cohort studies.

Ad-lib = *ad libitum* Chow diet; HF = high fat diet; CONT = Controls, fed standard Chow; PLHF = pregnancy and lactation high fat, that is, dams fed a high fat diet during pregnancy and lactation only; MHF = maternal high fat, that is, dams fed a high fat from weaning and throughout pregnancy and lactation.

2.2.5 Birth

At birth, the following measures were recorded for each litter:

- Litter Size
- Sex Ratio (male : female)
- Birth weight of each pup
- Body length – Nose to Anus (NA), and Nose to Tail (NT)

On the second day after birth each litter was standardised to 8 pups (4 males and 4 males) to standardise nutrition until weaning. Litters born with <8 pups were not used due to the confounds of altered neonatal nutrition and neonatal overfeeding associated with reduced litter size.

2.2.6 Lactation

During lactation the various treatment groups continued to receive manipulation of their nutrition according to the schedule outlined above. This required that Chow intake of the Controls be measured daily, so that 50% Undernutrition could be calculated for the UNL and the UNPL groups. Measurement of food consumption also allowed for internal checks of consistency between groups undergoing similar treatments, and for calculation of caloric intakes.

Pup growth and body weight was monitored throughout lactation. Pups were weighed on the first and second days of life, and every second day thereafter until weaning occurred at 22 days of age (day P22, where P = 'postnatal' age in days).

Following weaning, dams were euthanased (decapitation following anaesthesia with pentobarbitone, IP, 60mg/kg). Bloods were collected from the dams, kept on ice and spun down within 2 hours. Plasma supernatants were frozen and stored at -20°C for later analysis.

2.3 Evaluation of offspring and postnatal development

2.3.1 Weaning

At 22 days of age (P22) the offspring were weaned and housed as pairs for the remainder of the study. In total 338 pups were taken forward, housed in 169 cages across the 2 nutritional models. With 4 males and 4 females from each litter, the experimental design enabled us to place 2 animals from each gender on 1 of the 2 postnatal diets, as detailed below. This allowed us to minimise "litter of origin" effects on interaction with levels of postnatal nutrition.

2.3.2 Post-Weaning

The pups from each litter were randomised into pairs, which were placed into 1 of 2 postnatal dietary groups: either *ad libitum* Chow or *ad libitum* HF.

Total sample size *n* of rats assigned to each post-weaning dietary treatment group is outlined in Table 2.3 below. Since each litter generated two offspring per post-weaning dietary group, the biological replicate is *n*/2, i.e. a sample size *n*=18 is derived from 9 litters.

Table 2. 3 Numbers of offspring per treatment group

	Post-Weaning Dietary Group			
	Male Offspring		Female Offspring	
	AD Chow	AD HF	AD Chow	AD HF
Controls	18	18	18	18
UNP	14	14	14	14
UNPL	12	12	12	10
UNL	12	12	12	12
MHF	16	14	16	16
PLHF	14	12	14	14

AD = *ad libitum*, HF = high fat
UNP = dams fed 50%UN during pregnancy only; UNPL = dams fed 50%UN during both pregnancy and lactation; UNL = dams fed 50%UN during lactation only; MHF = dams fed a high fat from weaning and throughout pregnancy and lactation; PLHF = dams fed a high fat diet during pregnancy and lactation only.

Spanning the 2 nutritional models, there were a total of 24 treatment groups with a minimum of 10 animals per group. Control groups contained 18 animals each.

2.3.3 Post-Weaning Growth & Development

The weaned offspring were weighed every day from days P22 – P30, and thereafter every third day (ie, on days P33, 36, 39, 42, etc.) This continued until P132 at which point the growth curves were starting to plateau and the weighing schedule was reduced to once every 6 days (i.e. days P132, 138, 144, etc.). Additionally, each animal was weighed on the morning of its cull following an overnight fast.

2.3.4 Food Consumption & Energy Intake

Food consumption was measured every day from weaning (P22) until P30. The amount of remaining diet in the hopper was weighed and recorded, then the food hopper was topped up to the baseline amount in preparation for the next day. From these data diet consumption (weight of food, unadjusted in grams) and energy intake (adjusted to kcals/g bodyweight) were calculated.

After P30 food consumption was measured at defined periods from early post-pubertal through to adulthood (P42-43, P63-64, P96-97, P147-153). Food was replaced entirely every 6 days and old diet discarded (according to the respective diet manufacturers, diets were stable for at least 9 days in food hoppers at room temperature).

2.3.5 Evaluation of Onset of Puberty

From age P27, all offspring were checked daily for markers of reproductive maturity: vaginal opening and canalisation in females and balanopreputial separation in males. The same two investigators performed all pubertal checks (Graham Howie and Dr Deborah Sloboda).

2.3.6 Body Composition

Body composition was assessed using dual energy x-ray absorptiometry (DEXA, Lunar Hologic, GE Medical Systems, Waltham, USA). The DEXA instrument differentiates body weight into the components of lean soft tissue, fat soft tissue and bone, based on the differential attenuation by tissues of two levels of x-rays. Scan time is approximately 2 minutes, with no restraining devices/holders used and the method is completely non-invasive. Rats were initially anaesthetised in clear perspex chambers using 4% halothane and scavenging attachment. Once anaesthetised, rats were transferred and maintained under light (2%) anaesthesia using a nose-cone device. Rats were then scanned and then returned to their home cage. The whole procedure took

approximately 5 minutes per animal. There was no effect of the DEXA procedure on body weights or food intakes in the period following the scan.

Age of males at DEXA:	mean = 153 days, range = 151-156 days
Age of females at DEXA:	mean = 155 days, range = 151-162 days

2.3.7 Tissue Collection

Due to logistical considerations and the nature of the staggered mating protocol, tissue and blood collection was undertaken over a 3 week period so as to minimise variance in the mean age at cull. On each cull day, experimental groups were rotated so as to avoid time-of-day effects on sample collections, i.e. the mean time of cull was similar across all experimental groups and all culls were undertaken in the morning.

Animals were fasted overnight, anaesthetised by intraperitoneal pentobarbitone injection (60mg/kg), and killed by decapitation. Of a total of 338 animals, only 2 died (0.6%) under anaesthesia, and samples were not collected from these animals.

Age of females at cull:	mean = 173 days,	range = 169-177 days
Age of males at cull:	mean = 181.8 days,	range = 176-186 days

A tail snip was used to collect a fasting glucose measurement at the time of cull. (Optium Xceed, Abbott Diabetes Care, Victoria, Australia). Trunk blood was collected into heparinised vacutainers and stored on ice until centrifugation and separation of the plasma supernatant for later analysis. The pancreas was immediately dissected and snap frozen for later molecular analysis. Other tissues (heart, liver, kidneys, gonads, adipose, adrenal, spleen, brain) were collected and weighed. Although beyond the scope of this thesis, the other tissues were banked for later molecular and/or histological analysis by other researchers in independent studies.

2.4 Laboratory analysis

2.4.1 ELISAs

Commercially available ELISA kits were sourced for plasma analysis. A rat-specific leptin ELISA and an ultra-sensitive rat insulin ELISA were purchased from CrystalChem Inc (IL, USA, http://www.crystalchem.com, with catalogue references #90040 and #90060 respectively). All kits were from the same batch and the intra- and inter-assay coefficients of variation were <5% and <9% respectively for both kits.

2.4.2 Tissue Disruption

Whole pancreas was ground to powder in liquid nitrogen using a manual mortar and pestle.

Throughout disruption, every effort was made to prevent cross-contamination of the ground tissues. All equipment was freshly autoclaved before use. The mortar and pestle were scrubbed out with 70% ETOH between samples, then thoroughly dried with autoclaved tissues. Forceps received similar treatment. Gloves were frequently sprayed with 70% ETOH spray and replaced between samples. The pulverising station had a fresh layer of autoclaved tinfoil sheeting laid down for each sample.

The powdered pancreas was divided into two pre-cooled and labelled tubes – one to be used for RNA/DNA extraction, and the other for storage of backup tissue. The tubes were immediately placed back on dry ice, and then securely stored at -80°C.

2.4.3 Tissue Preparation

Betamercaptoethanol (β-ME) was used to extract the RNA and DNA material from the homogenised tissue. β-ME inactivates RNase and DNase enzymes so that intact RNA and DNA can be isolated.

The β-ME solution was added to approximately 25mg of ground pancreatic tissue, and they were then homogenised together for 10-20 seconds.

Maintaining purity of sample tissue

The spinning probe of the homogeniser was replaced with a freshly autoclaved probe for each specimen. After its singular use the probe was spun in clean MilliQ water for 10-20 seconds, then again in 100% ETOH. At the end of each session the probes were dismantled and any visible tissue remnants rinsed off with ETOH. The probes were soaked overnight in 100% ETOH, then autoclaved in sealed pouches for re-use.

2.4.4 RNA/DNA Extraction

The procedure used to extract RNA and DNA* from the homogenised pancreatic tissue was a combination of two processes from two commercial kits (1x RNA plus 1x DNA kit):

RNeasy MICRO Kit, catalogue #74034,

DNeasy Blood and Tissue Kit, catalogue #69504

Kits were sourced from Qiagen (www.qiagen.com) and purchased through:

Thermo Fisher Scientific, 244 Bush Rd, Albany, Auckland 0632

www.thermofisher.co.nz

(* Although beyond the scope of this thesis, DNA was extracted and stored for future independent studies with the possibility of looking at possible alterations in DNA methylation in selected genes of interest.)

The isolation and collection of the RNA/DNA nucleotide sequences was as per manufacturer's instructions:

- **Centrifugation** The cellular debris resulting from tissue disruption and homogenisation was spun to the base of the tube by centrifugation. Special care was taken to pipette up only the supernatant, and not the pellet of debris from the bottom of the tube. If the pellet was disturbed then the tube was re-spun.

- **Selective Binding and Filtration** The spin-column membranes of the Qiagen kits are designed to isolate and bind the nucleotide material. Contaminants (such as salts) could then be rinsed away by various buffer solutions as supplied and the flow-through after centrifugal spinning was discarded.

- **Elution** After washing, the desired nucleotide bound to the spin column membrane was eluted in RNase-free water, and spun off the membrane to be retained in the bottom of the tube for collection and storage.

2.4.5 Nanodrop

The concentration (ng/µl) of RNA and DNA was calculated by measuring the spectophotometric absorbance at 260nm (A_{260}). The purity of the sample was determined by the ratio between readings at 260nm and 280nm (A_{260}/A_{280}) - particularly with respect to protein contamination, since protein (such as the aromatic amino acids) tends to absorb at 280nm. A sample with an A_{260}/A_{280} ratio of 2.00 is 100% pure nucleic acid with no protein contamination. In this study we accepted an A_{260}/A_{280} ratio range of 1.75- 2.25 (the lower value was more critical than the upper one).

A second measure of nucleic acid purity was the ratio between 260nm and 230nm (A_{260}/A_{230}). An ideal A_{260}/A_{230} ratio for 'pure' nucleic acid is 2.00. A lower ratio than expected is suggestive of contaminants that absorb at the 230nm level, such as salts, phenolate and other organic compounds. However, a good sample typically has an A_{260}/A_{230} ratio that is a little higher than the A_{260}/A_{280} ratio. The presence of contaminants was further revealed in distortion of the typically smooth Absorbance/Wavelength curve on the Nanodrop display.

The Nanodrop machine was calibrated before each sample, using the dilutant each product was contained in – RNase-free H_2O for RNA samples, and AE buffer for DNA. If samples proved to be of very high concentrations, that is >2000 ng/µl, samples were diluted 1:1 or 2:1 and the assay was repeated.

2.4.6 Agarose Gel Electrophoresis

Agarose gels were run to test the quality of the extracted mRNA. A 1% gel was mixed and ethidium bromide (EtBr) added (8µl per 100ml of buffer). EtBr was picked up by the nucleic acid as it progressed through the gel under electrophoresis, and then fluoresced under ultraviolet light, producing a bright band that revealed the number and size of the RNA fragments. Typically two bands of fluorescence were seen: one for mRNA and another for tRNA (which, as a smaller molecule, travelled further down the gel toward the positive electrode).

A standardised amount (2µg) of RNA was placed in each gel well. The volume of elution that contained 2µg weight of RNA was calculated from the concentration as determined by Nanodrop. The final total volume of liquid to be placed in each well of the gel was 12µl. This final total volume consisted of three components: the volume of RNA calculated to contain 2µg; 2µl of loading dye; plus enough MilliQ water to make up the difference to 12µl.

Elution liquid that did not reveal two clean bands of RNA fluorescence on the agarose gel was discarded, and a re-extraction of RNA was performed from the spare ground pancreatic tissue held in storage.

2.4.7 cDNA Synthesis & Amplification

This step was a standard 'hot-start' PCR which used the Reverse Transcriptase enzyme (MMLV-RT) to synthesise cDNA from the extracted mRNA.

The protocol used was:

- Place tubes (= H_2O + Random Primers + mRNA) into PCR machine for 5 mins at 70°C to melt any secondary structures that form.
- Snap cool tubes in ice for 2 mins to prevent secondary structures from re-forming.
- Cool PCR machine down to 37°C.
- Add master mix to tubes (= Reverse Transcriptase + dNTPs + MMLV Buffer), while tubes are still on ice.
- Place tubes back into PCR machine, set to cycle:

  1st hold: 96°C for 5 mins

  28 cycles: 96° for 30 secs, then 58° for 30 secs, then 72° for 1 min

  2^{nd} hold: 72° for 5 mins, then held at 4° until retrieved

2.4.8 cDNA PCR with Housekeeping Gene

The purpose of this step was to check the presence of cDNA following the synthesis process. Primers for a known 'housekeeping gene' (HKG) were added to a small amount of the synthesised product and put through a PCR amplification process, then run on an agarose gel under electrophoresis against a DNA ladder to check if the amplicon (the HKG product) was the stipulated size.

Following investigation of the expression of a range of common HKGs, HPRT proved to be the most reliable housekeeper as others examined were significantly affected by experimental conditions i.e. level of maternal nutrition. Therefore we used HPRT as our HKG, with custom-designed forward and reverse primers, supplied by Invitrogen, (www.invitrogen.com).
HPRT produced a single band at the 200bp level of the DNA ladder.

The enzyme used was Go Taq Polymerase Flexi, from Promega, catalogue # M8291

The PCR machine was set for the following thermo-cycling programme, and a representative selection of samples was checked:

5 mins at 94°C
30 x cycles of: 94°C (30 secs) then 58°C (30 secs) then 72°C (30 secs)
5 mins at 72°C
Hold at 4°C

2.4.9 Primer Design

Three avenues were utilised for obtaining suitable primers for our real-time gene expression studies:

1) Self-designed (7/15 genes investigated)
2) Commercially sourced primers (7/15 genes studied)
3) Primer sequences extracted from the background literature and prepared commercially (1/15 genes studied)

When designing primers the following criteria were followed:

- GC% - about 50%. An increase in Gs&Cs will increase strength of binding, but too much GC binding power can promote non-specificity of binding. Similarly, long stretches of a single base (≥ 4) were avoided, especially of C or G – such homopolymeric runs can lead to ambiguous binding and misprimed elongation, an effect know an 'slippage' (290).
- Amplicon length – about 100-250 base pairs (bp). An amplicon such as this, that is relatively short, permits faster and more efficient amplification. It is also easier to run on a gel – a shorter sequence is able to travel further through the agarose and separate nicely from any longer products.
- Max 3' self-complementarity setting was always kept at 0, to reduce the chance of the primer binding to itself and forming dimers.
- All potential primer sequences were BLASTed to make sure that they were unique to the gene of interest and would not amplify any other portion of DNA.
- If the target gene was composed of several exons, then a primer was designed that overlaid an exon-exon boundary. This makes the primer exclusive to cDNA, and it will not anneal to gDNA.

2.4.10 qPCR – Real-time PCR, Quantification of Gene Expression

Quantitative PCR methods seek to estimate the amount of amplicon, typically with spectrophotometry, using fluorescent dyes. Often this is done only at the end of the amplification process. However, real-time PCR performs this estimate at each repetition of the thermo-cycling programme as the amplification process continues exponentially. The amplification curve is sigma shaped – from a phase of no product visible, to a steep rising phase of exponential amplification, finishing with a plateauing off as dNTPs and other reagents are exhausted.

We used SYBR-Green (Invitrogen) which is a commercially made pre-mixture of the polymerase enzyme, the fluorescent dye and other necessary reagents. The SYBR-Green dye binds to double-stranded DNA, which substantially increases its fluorescing signal – thus the amount of fluorescence is proportional to the amount of DNA amplification.

We used an Applied BioSystems ABI PRISM 7900HT Version 2.3 Sequence Detection System, with the real-time amplification / quantification cycler set to the following protocol:

Stage 1:	50° for 5 mins
Stage 2:	95° for 2 mins
Stage 3:	95° for 15 secs, then 60° for 1 min = 40 x Repeats
Stage 4:	95° for 15 secs, then 60° for 15 secs, then 99° for 15 secs

A standard curve that exhibited 100% amplification efficiency would have a slope of -3.32. However, such absolute efficiency is rare, due to factors such as amplicon length, GC content, and quality of enzyme and other reagents. Thus a slope of between -3.10 and -3.58 was accepted (291).

Similarly, an R2 of 1.0 is ideal. Careful technique and the exclusion of any STDs whose Ct values are deviant (i.e., do not meet the criteria listed below) made an R2 of >0.99 achievable.

A pilot real-time PCR of a small set of samples at different dilutions was always performed with each new set of primers. This was to determine the optimal cDNA dilution to use, so as to land the majority of the C_t values in the mid range of the Standard Curve.

Consistency & Accuracy All samples were loaded onto the 384-well real-time plate in triplicate. Once amplified and detected by the real-time machine, C_t values for each triplicate were compared and only accepted if they met the following criteria:

- C_t values must be within 0.5 of each other
- Melting curves must be single peaked (ie, without evidence of primer-dimers or other non-specific amplification products)
- Shape and peak temperature of melting curves must conform to curves generated by the Standards
- At least two of the three wells must meet these criteria for a mean of the sample to be generated.

Chapter 3: Maternal undernutrition during critical windows of development results in differential and gender specific effects on postnatal adiposity and related metabolic profiles in adult rat offspring

3.1 Introduction

Impaired fetal growth is a major cause of perinatal morbidity and has long-term clinical consequences. Epidemiological evidence linked low birth weight to an increased risk of developing adult diseases including type 2 diabetes, hypertension and cardiovascular disease (4, 292-294). Poor fetal growth also remains the predominant factor in determining perinatal morbidity and mortality and alterations in perinatal growth may have long-term consequences for somatic growth and development, endocrine and metabolic function and cardiovascular status. This association in human populations has been explored in various animal models (119, 163, 295-297) where it has been demonstrated that nutritional restriction during pregnancy could indeed produce offspring with reduced birth weight which went on to exhibit accelerated weight gain and obesity in adulthood (particularly in the presence of an *ad libitum* postnatal diet), and displayed symptoms that were similar to that of the human metabolic syndrome.

The proximate causes of human obesity and the metabolic syndrome are typically thought to be changes in contemporary diet and lifestyle. Western dietary habits have tended toward over-eating, plus increased consumption of energy-dense or high-caloric foods such as fats, whereas modern lifestyles have become less active, more sedentary. These behavioural changes are probably due to environmental influences: the rapid rise in these pathologies precludes the causative agent being genetic. The DOHaD hypothesis, however, offers an original contribution to understanding this phenomenon: developmental programming links formative early life events to later life adult metabolic function. The DOHaD model speculates that intrauterine cues allow the fetus to make predictive adaptations which not only aid immediate survival, but also shape physiological development in preparation for an anticipated postnatal environment. However, dissonance results when the postnatal environment is different from that anticipated, leading in a mismatch between prenatal predictions and postnatal reality. This may be due to misinterpretation of prenatal cues or to a change in the anticipated postnatal environment. Where mismatch occurs the predictive adaptive responses (PARs) may have ill-equipped the phenotype for the ensuing adult environment, producing an increased vulnerability to ‘lifestyle’ diseases.

These effects (such as the tendency to obesity) may then be transmitted across generations (298-300).

Implicit in the PARs hypothesis is the role of 'catch-up' growth (4, 295, 301) – a classic case of 'mismatch' between malnutrition before birth and a normal or increased level of nutrition after birth. This situation is relatively easily achieved experimentally: firstly, the pregnant rat dam is placed on restricted feeding during gestation to produce IUGR pups. Secondly, post-parturition nutritional mismatch can be achieved in a number of ways: the dam can be returned to *ad libitum* feeding during lactation (302-304); or the newborn can be cross-fostered to another well-nourished mother (296, 301, 305); or the litter size could be reduced to make more breast milk available to the remaining animals (130, 162); or the offspring could be artificially fed by intra-gastric tube (125, 126). All these methods of nutritional mismatch have reliably produced elements of the metabolic syndrome in offspring. Indeed, it has been asserted that fetal undernutrition alone will not programme offspring obesity without the addition of increased postnatal nutrition (303). The design of the current study allowed us to test this assertion.

The magnitude of programming effects is known to vary depending on the type and the timing of early malnutrition. The current study is balanced to probe the effects of undernutrition during pregnancy *versus* lactation, with or without 'catch-up' nutrition during lactation, and to uncover any gender effects in male *versus* female offspring. Moreover, we have added a post-weaning dietary manipulation whereby after lactation all cohorts are randomised to be fed *ad libitum* either Chow or a high fat (HF) diet. This will allow us to examine the relationship between our various offspring phenotypes (programmed through maternal undernutrition during differing developmental windows) and a calorie dense diet consumed later in life.

Previous studies utilising altered maternal nutrition during discrete critical periods of development have either directly examined low protein effects (306) or commenced maternal undernutrition midway through gestation (307), thus missing the critical pre-implantation period known to give rise to metabolic abnormalities in offspring (308, 309). In addition, the role of a post-weaning HF diet to further exacerbate the dietary "mismatch" has not been well explored. The present study therefore investigated the effect of moderate maternal global undernutrition during pregnancy and/or lactation to examine postnatal metabolic sequelae in male and female offspring following well-defined periods of early life nutrient deprivation.

In addition we examined all offspring for age and body weight at pubertal onset. In humans, girls with low birth weights who experienced accelerated growth gain during childhood, entered

puberty at a younger age. This has been widely demonstrated in a number of countries (280, 310-315). These effects may be restricted to females: several studies have found no association between low birth weight or accelerated postnatal growth, and pubertal onset in males (316, 317). However, investigations of pubertal onset for boys are less common than for girls, simply because there is no marker of pubertal onset in males that has proved as straightforward as menarche has for girls. Although the primary focus on this thesis is on growth and metabolism, the current study also provided an opportunity to examine sex-specific effects of maternal undernutrition on pubertal onset in offspring.

The predictive adaptive response (PAR) hypothesis argues that the developing organism is sensitive to intrauterine cues such as the prenatal nutritional environment, and these cues function as 'predictors' that 'forecast' the postnatal nutritional environment. Such cues help shape the development of the fetus, to fit it for metabolic success in mature life. Fitness from an evolutionary perspective also involves successful reproduction, thus it makes sense that intrauterine cuing might also shape reproductive maturation (318, 319). Part of an adaptive response to early life nutritional deprivation might therefore be expected to be an acceleration in the age of reproductive ability, provided that the organism can metabolically sustain earlier reproduction without further compromising its own viability. Rats exposed to maternal malnutrition and thus programmed for increased adiposity might well be suitable candidates for an early onset of puberty.

The aims of the current study are: to investigate the differential programming effects of undernutrition during pregnancy and/or lactation; to compare programming in male *versus* female offspring; to investigate the PAR hypothesis of match *versus* mismatch across the two windows of gestation and lactation; to check whether an adverse early nutritional environment affects reproductive maturation, and whether this is different in males *versus* females.

In the current study we have utilised a model of 50% maternal nutrition. This is in contrast to the 70% undernutrition model that this laboratory has previously applied (123, 124, 296, 297). It is an additional aim of this study to examine if the programming effects that have been reliably produced by the earlier 70% undernutrition model will be as equally evident in a more moderate model of maternal undernutrition.

3.2 Materials and Methods

We have already published some initial findings from this particular cohort (320, 321). The animal model utilised and the endpoint indices measured were described in detail in Chapter 2, but are reiterated here in brief.

Animal Work Female Wistar rats (120 days of age) were time-mated using an estrus cycle monitor (Fine Science Tools, USA). Upon confirmation of mating, females were randomly assigned to 1 of 4 maternal dietary groups:

1. **CONT** (= Controls): Females maintained on an *ad libitum* diet of standard Chow throughout pregnancy and lactation
2. **UNP** (= Undernutrition during pregnancy only): Females fed standard Chow at 50% of Control intake throughout pregnancy
3. **UNL** (= Undernutrition during lactation only): Females fed standard Chow at 50% of Control intake through lactation alone
4. **UNPL** (= Undernutrition during both pregnancy & lactation): Females fed at 50% of Control intake throughout both pregnancy and lactation.

All pregnant dams were weighed and had food intakes measured daily throughout pregnancy and lactation.

Following birth, pups were weighed, had body lengths recorded and litter size was randomly adjusted to 8 pups (4 males and 4 females) to ensure standardized nutrition until weaning. Non-assigned pups were killed by decapitation at postnatal Day 2 (P2) and plasma samples pooled for later analysis. Pups were weighed every three days until weaning.

After weaning (P22), dams were fasted overnight, anaesthetised with sodium pentobarbitone (60mg/kg, IP) and killed by decapitation. Maternal plasma samples were collected for insulin and leptin analysis.

At weaning, all offspring were weight matched within maternal dietary treatment groups and placed on a postnatal *ad libitum* diet of either the standard rat Chow or a HF diet. Male and female offspring were housed 2 per cage (2 per litter/gender/maternal background/postnatal diet) until the end of the trial (P160). (n = 12-18 per group.)

From postnatal days P27 – P40, pubertal onset was checked daily in male and female offspring using standard techniques: vaginal opening and canalisation in females, and balanopreputial separation in males.

At age P150, animals had body composition quantified by dual energy x-ray absorptiometry (DEXA, GE Lunar Prodigy, Waltham, USA) scanning while under light isoflurane (2%) anaesthesia. (For logistical reasons, not all animals per cohort were scanned. n = 8-10 per group.)

At age P160, animals were fasted overnight, anaesthetised with sodium pentobarbitone (60mg/kg, IP) and killed by decapitation. Tissues (liver, heart, kidneys, spleen, gonads, adrenals, brain and retroperitoneal fat pad) were weighed and promptly frozen at -80°C. Blood was collected into heparinised Vacutainer tubes and stored on ice until centrifugation, with plasma supernatant stored at -20°C for future analysis.

The Animal Ethics Committee of the University of Auckland approved all animal experimentation (R402).

Plasma analyses Leptin and insulin concentrations in dams and offspring were analysed using commercial rat-specific ELISAs (Cat#90040 and 90060 respectively, CrystalChem, Downers Grover, IL, USA). Fasting plasma glucose concentrations were measured using a glucose meter at the time of cull (Roche AccuChek). Plasma insulin:leptin ratios were calculated as a proxy for adipoinsular axis function (235).

Statistical Analyses Data were analysed using the SigmaStat 3.1 statistical package (SYSTAT Software Inc, IL, USA). Different levels of ANOVA were utilised as appropriate: where One Way ANOVA was used, maternal diet or gender were the sole factors; where Two Way ANOVA was used, the factors were maternal diet and postnatal diet; occasionally a Three Way ANOVA was used, with maternal diet, postnatal diet plus gender as factors. Repeated measures of ANOVAs were used to analyse growth curves and energy intake curves. The Holm-Sidak method was used for all pairwise multiple comparison procedures, unless otherwise indicated. Statistical significance was accepted at the $P<0.05$ level. All data are presented as means ± S.E.M. unless otherwise stated.

3.3 Results

3.3.1 Maternal weights

Pregnancy All dams in this undernutrition study were raised on standard Chow from weaning; all entered pregnancy at similar body weights. CONT and UNL dams were fed *ad libitum* Chow through pregnancy and had parallel weight gain (Weight gain curves, CONT *versus* UNL dams = NS, P=0.60). The UNP and UNPL dams also experienced identical dietary treatments during pregnancy (50% of CONT dams) and this maternal undernutrition resulted in similar reduced body weight gain, where weight declined until day G15, from which point there was a slow increment in weight gain until birth (Figure 3.1). There was no difference in length of gestation between any of the maternal groups.

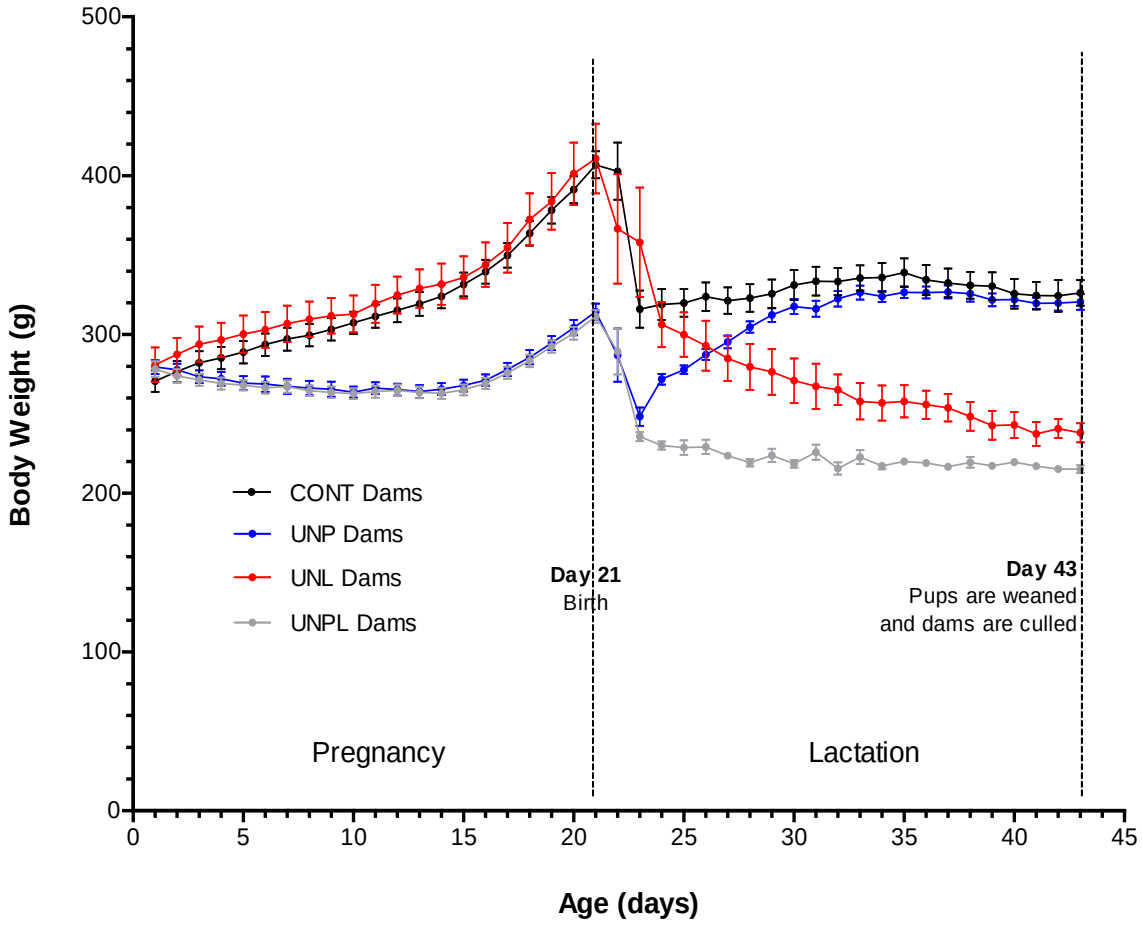


Figure 3.1: Body weights during pregnancy and lactation of chow-fed control dams (CONT), dams fed 50% undernutrition throughout pregnancy (UNP), dams fed 50% undernutrition throughout lactation (UNL), or dams fed 50% undernutrition during pregnancy and lactation (UNPL). Data are means ± SEM, *n* = 6-8 dams per group.

Lactation During lactation, the body weight curve of the CONT dams remained stable, with no significant weight change across this period. UNP dams, having been returned to *ad libitum* Chow, rapidly regained weight, so that by Day 27 their mean body weight was similar to the CONT group. The body weights of the UNPL dams remained reduced but stable through lactation: they were the lightest of the treatment groups. The UNL dams, transitioned to 50% undernutrition during lactation, steadily lost weight until by Day 37 their mean body weights are no longer significantly different from the UNPL dams. (Figure 3.1)

3.3.2 Energy intake of dams during pregnancy and lactation

Pregnancy Energy intake immediately rose as soon as the rat dam became pregnant. There was no significant difference between the overall energy intake curves for CONT *versus* UNL dams. Energy intake remained constant at the higher level of pregnancy from days 1-19, before dropping steeply in the last two days before parturition. The energy intake curves of the UNP and UNPL dams are tightly aligned – because they both received the same 50% undernutrition, calculated directly against the CONT's consumption. Energy intake appeared to climb steadily in these two groups of undernourished dams. However, this is simply an artefact of the computation (kcals per gram of body weight), because body weight was falling in UNP and UNPL animals. (Figure 3.2).

Lactation In all groups of dams, energy intake reached its lowest level on the day of the pups' birth, then climbed steeply, continuing to increase throughout the lactation period. By the second day of lactation it had surpassed the steady levels during gestation. By the end of lactation it was four-fold what it had been during gestation. There were clearly very high energy demands on lactating dams, and these increased as the pups grew larger.

As expected, the energy intake curves for the UNL and UNPL dams remained lower than the CONT and UNP dams ($P<0.05$, Two Way Repeated Measures ANOVA, with factors Maternal Diet and Age.) Energy intake for UNL and UNPL dams became similar from day 39.

The curve for the UNP dams is noteworthy for the large upward spike seen on Day 23, the first day after birth. This was the day when these dams were returned to *ad libitum* Chow after being on 50% UN throughout gestation. Their physical food consumption that day was nearly twice that

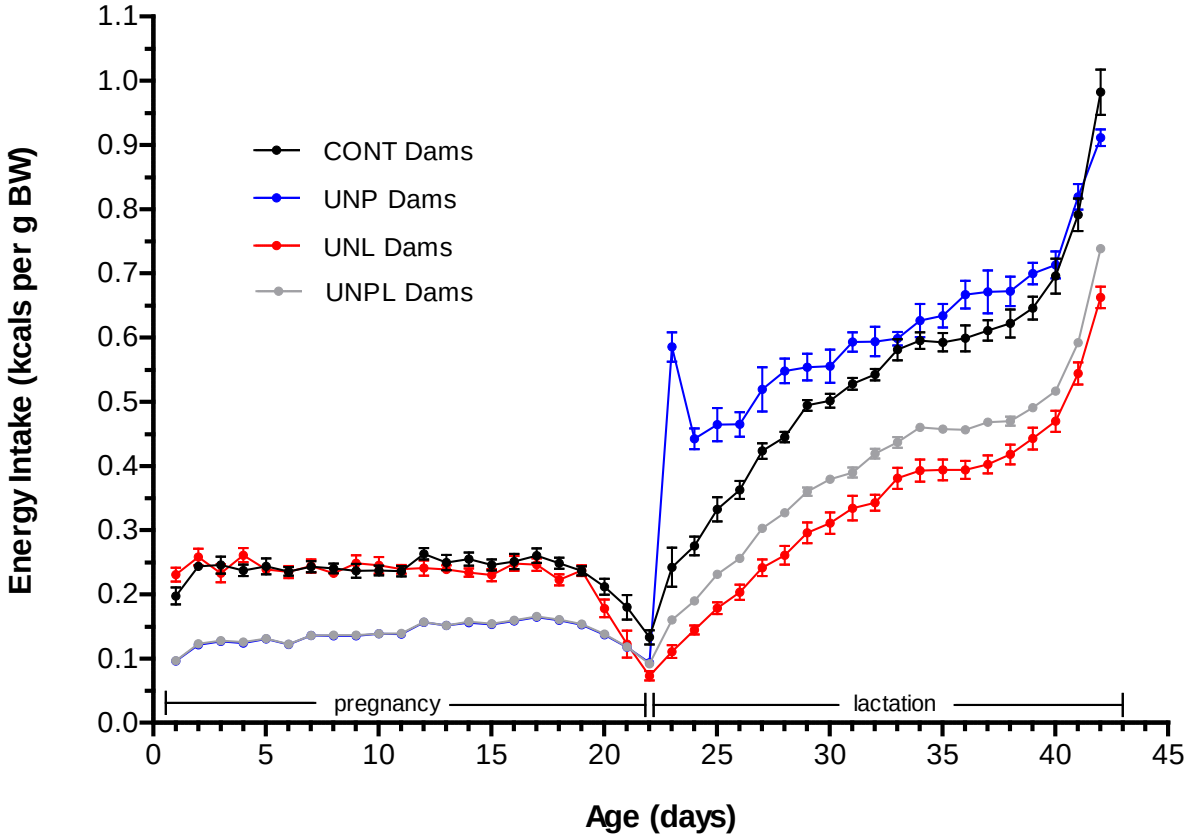


Figure 3.2: Energy intake (kcal/g body weight) during pregnancy in chow-fed control dams (CONT), dams fed 50% undernutrition throughout pregnancy (UNP), dams fed 50% undernutrition throughout lactation (UNL), or dams fed 50% undernutrition during pregnancy and lactation (UNPL). Data are means ± SEM, n = 6-8 per group.

of the CONT dams (in weight, data not shown), and because they were relatively light in body weight, this translated into a marked increase in relative caloric intake.

As the UNP dams gained weight (their body weight approached the CONT dams by the end of the lactation period), their energy intake curve became statistically no different to the CONT dams' curve by day 37, in the last week of lactation.

But note: the intake in the last few days may be confounded by intake from the weaners who at the later stages of lactation are able to consume the standard control diet in addition to suckling.

3.3.3 Maternal plasma insulin and leptin

There was no statistical difference in maternal fasting plasma glucose (data not shown) or fasting plasma insulin at the end of lactation (Table 3.1). Maternal plasma leptin was significantly decreased in UNL and UNPL dams compared to UNP dams (Table 3.1). Due to the confounders of stressors, blood samples were not taken at the time of birth from dams.

Table 3.1: **Maternal plasma concentrations of insulin and leptin, and insulin:leptin ratio at the end of lactation (P22)**

Group	Maternal Insulin (ng/ml)	Maternal Leptin (ng/ml)	Maternal Insulin:leptin ratio
CONT	1.91 ± 0.4	1.71 ± 0.20	1.14 ± 0.21
UNP	1.33 ± 0.3	2.16 ± 0.26 [c, d]	0.61 ± 0.12 [d]
UNL	2.87 ± 1.1	1.09 ± 0.15 [b]	2.78 ± 0.96
UNPL	3.91 ± 1.1	1.07 ± 0.06 [b]	3.62 ± 1.02 [b]

Data are means ± SEM, *n* = 6-8 per group.
CONT = control; UNP = dams fed 50% undernutrition throughout pregnancy; UNL = dams fed 50% throughout lactation; UNPL = dams fed 50% undernutrition during pregnancy and lactation.
Different letters denote significant differences between groups: [a] = different from CONT, [b] = different from UNP, [c] = different from UNL, [d] = different from UNPL. $P < 0.05$ (One Way ANOVA, with maternal diet as factor.)

In terms of the maternal plasma insulin:leptin ratio (a marker of altered adipoinsular axis function (235)), UNP dams had the lowest ratio and UNPL dams the highest – there was a significant difference between these two groups of animals. (Table 3.1)

3.3.4 Birth weights of offspring

Male and female birth weights were significantly reduced in the UNP and UNPL groups compared to CONT and UNL (Figure 3.2). Female pups tended to be slightly lighter at birth than male pups, in all groups. This difference between sexes was statistically significant in the UNP and UNPL groups, but not in the CONT or UNL groups. There was no difference in litter size or in sex ratio between the treatment groups (data not shown).

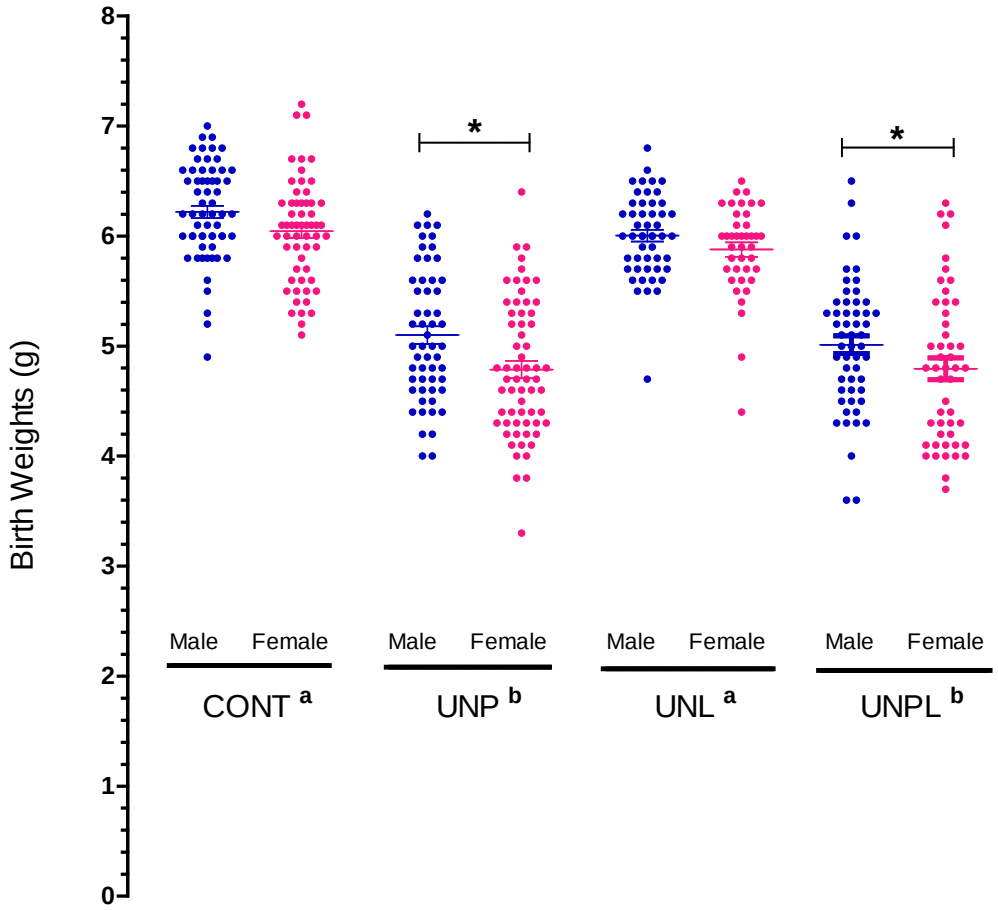


Figure 3.3: Birth weights of pups of chow-fed control dams (CONT), pups of dams fed 50% undernutrition throughout pregnancy (UNP), pups of dams fed 50% undernutrition throughout lactation (UNL), and pups of dams fed 50% undernutrition during pregnancy and lactation (UNPL), males (blue) and females (pink). Maternal 50% undernutrition during pregnancy (UNP & UNPL dams) resulted in a significant reduction in birthweight of pups, compared to *ad libitum* nutrition (CONT & UNL groups): $P<0.001$, all pups, genders combined, groups with different letters are significantly different from each other, One Way ANOVA with maternal diet as factor. Maternal 50% undernutrition (UNP and UNPL dams) also resulted in male pups whose birthweight was significantly heavier than female pups (*): $P<0.05$, Two Way ANOVA with maternal diet and gender as factors. This effect was not seen in dams who received *ad libitum* nutrition during pregnancy (CONT & UNL dams). Scatter plot has mean ± SEM bars, n = 40-63 per group.

3.3.5 Lengths at birth

Body length was measured at birth, both nose to anus (NA) measurement, and nose to tail (NT). The pups of UNP and UNPL dams were born significantly shorter in NA length than pups of CONT and UNL dams (Figure 3.4).

In all groups NA lengths at birth in males were increased as compared to females, but post-hoc analysis revealed that the NA difference between males *versus* females was only significant in the two groups that were undernourished during gestation (UNPs & UNPLs), not in the groups that received *ad libitum* nutrition (Figure 3.4).

Similar results were obtained for NT length, between groups and between sexes (data not shown).

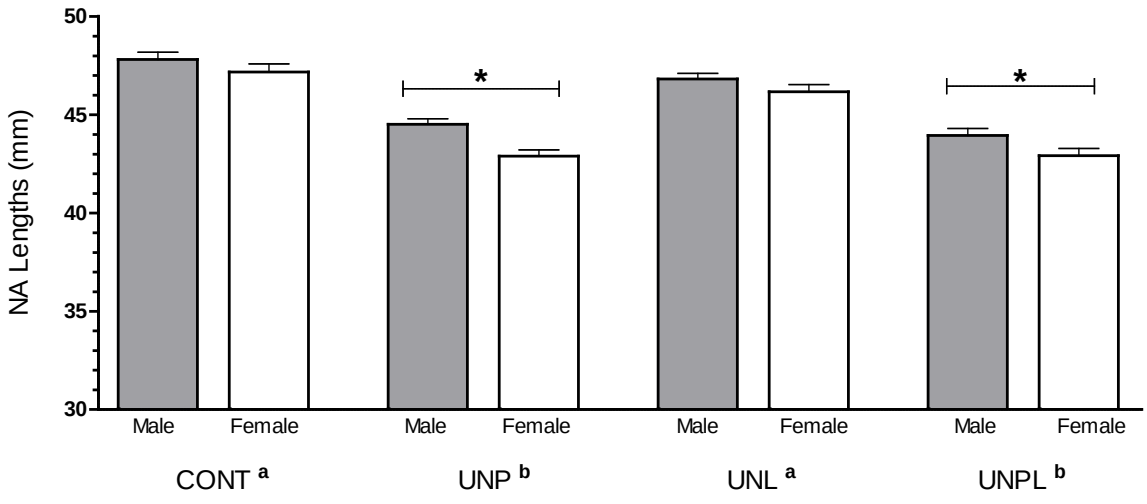


Figure 3.4: Nose to anus (NA) lengths at birth of pups of chow-fed control dams (CONT), pups of dams fed 50% undernutrition throughout pregnancy (UNP), pups of dams fed 50% undernutrition throughout lactation (UNL), and pups of dams fed 50% undernutrition during pregnancy and lactation (UNPL). Maternal 50% undernutrition during pregnancy (UNP & UNPL dams) resulted in a significant reduction in NA length of pups, compared to *ad libitum* nutrition (CONT & UNL groups), $P<0.001$, in both males and females, groups with different letters are significantly different from each other. Maternal 50% undernutrition (UNP and UNPL dams) also resulted in male pups whose NA length was significantly longer than female pups (*); Males *vs* Females: UNP, $P<0.001$; UNPL $P=0.012$. This effect was not seen in dams who received *ad libitum* nutrition during pregnancy (CONT & UNL dams); Males *vs* Females: CONT, $P=0.23$; UNL, $P=0.32$. Two Way ANOVA with maternal diet and gender as factors. Data are mean ± SEM bars. n = 40-63 per group.

3.3.6 Neonatal weight gain – males

CONT and UNL pups had similar birth weights. Likewise, UNP and UNPL pups had similar birth weights (reflecting the common nutritional histories of these two groupings of dams). UNP and UNPL pups were significantly lighter at birth than CONT and UNL pups.

UNP pups demonstrated catch-up growth between birth and 22 days of age, but they remained slightly but significantly lighter than CONT animals at the time of weaning.

UNL pups exhibited 'catch-down' growth. They were significantly lighter than the CONT and UNP pups at weaning, but heavier than the UNPL pups.

UNPL pups were born small and exhibited the least weight gain during lactation. They were significantly growth restricted compared to CONT and UNP offspring. (Figure 3.5)

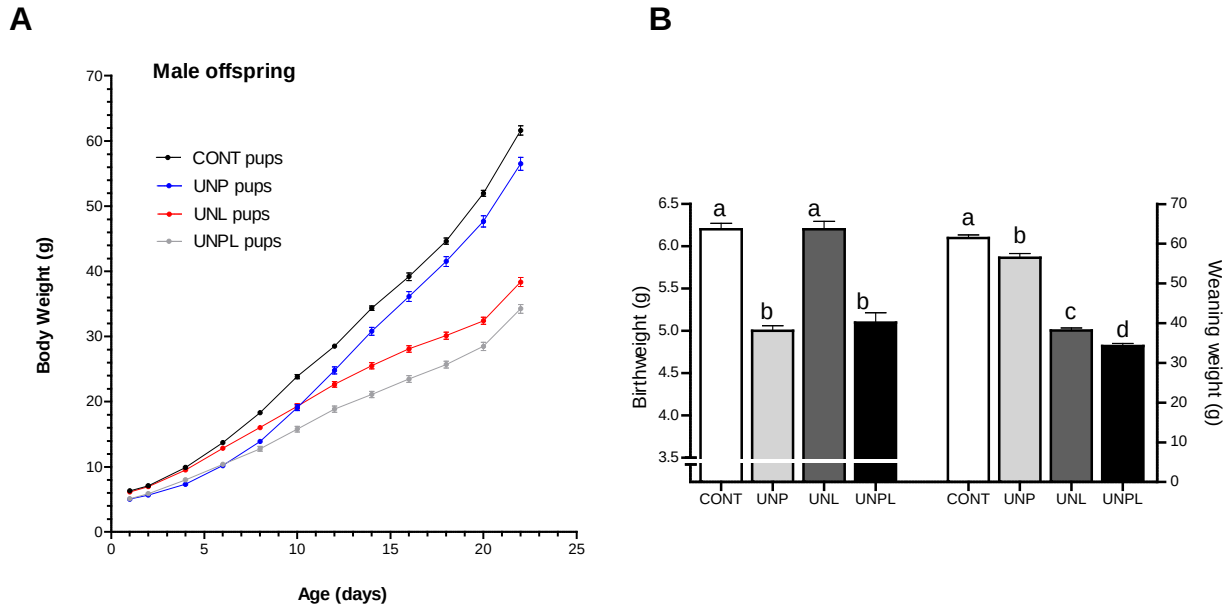


Figure 3.5: Weight gain from birth to weaning (P22) in male offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL).
The group curve of each offspring group was significantly different to other groups (A), $P<0.05$, Two Way repeated measures ANOVA, with maternal diet and age as factors. Maternal 50% undernutrition during pregnancy (UNP & UNPL dams) resulted in a significant reduction in birthweight of pups (**B**, left bar graph), compared to *ad libitum* nutrition (CONT & UNL groups), $P<0.001$, groups with different letters are significantly different from each other. By weaning (**B**, right bar graph), UNP pups showed catch-up growth and were nearly (but not significantly) the same weight as the CONT pups, whereas UNL pups had grown more slowly and were nearly (but not significantly) the same weight as UNPL pups, $P<0.001$, groups with different letters are significantly different from each other. Data are means ± SEM, n = minimum of 24 per group. One Way ANOVA with maternal diet as factor.

3.3.7 Neonatal weight gain – females

The pattern of weight gain during lactation in the female offspring was similar to the males.

The UNP pups exhibited catch-up growth, and their growth trajectory paralleled the CONT group, although there remained an overall significant difference between the growth curves for the two groups through this period.

The UNL pups exhibited 'catch-down' growth, and their growth trajectory paralleled the UNPL group, although their growth curve remained significantly different from the UNPL curve (and all other curves).

The UNPL pups had the lowest weight gain of all the groups.

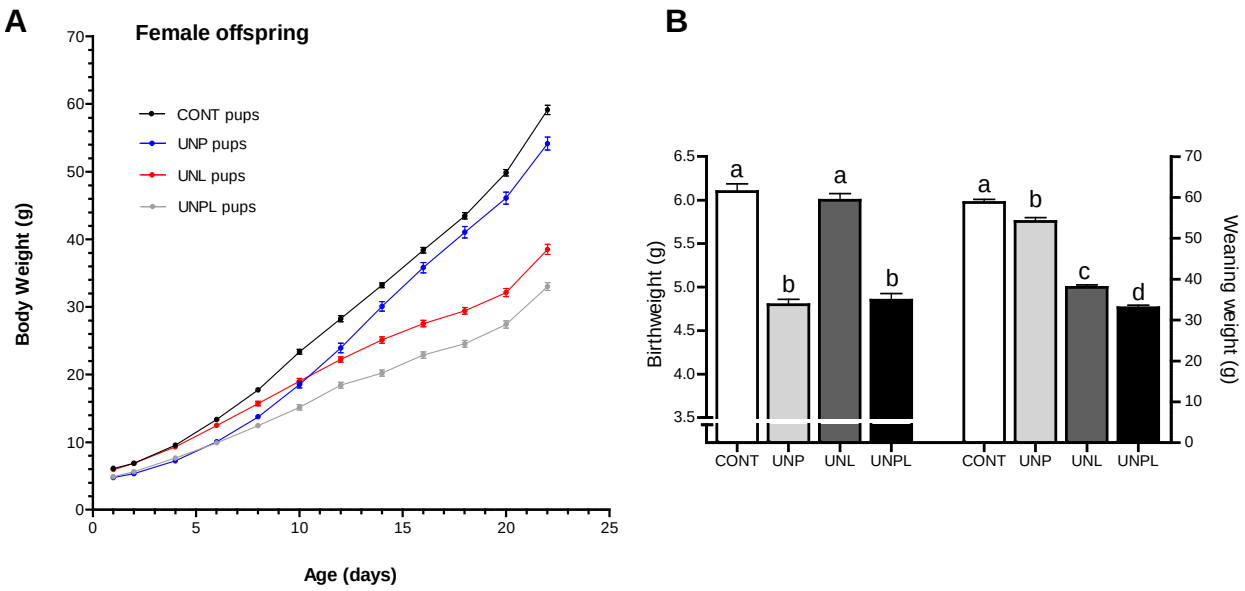


Figure 3.6: Weight gain from birth to weaning (P22) in female offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL). The group curve of each offspring group was significantly different to other groups (A), $P<0.05$, Two Way repeated measures ANOVA, with maternal diet and age as factors. Maternal 50% undernutrition during pregnancy (UNP & UNPL dams) resulted in a significant reduction in birthweight of pups (B, left bar graph), compared to *ad libitum* nutrition (CONT & UNL groups), $P<0.001$, groups with different letters are significantly different from each other. By weaning (B, right bar graph), UNP pups showed catch-up growth and were nearly (but not significantly) the same weight as the CONT pups, whereas UNL pups had grown more slowly and were nearly (but not significantly) the same weight as UNPL pups, $P<0.001$, groups with different letters are significantly different from each other. Data are means ± SEM, n = minimum of 24 per group. One Way ANOVA with maternal diet as factor.

3.3.8 Post-weaning growth

At weaning (P22) all groups of offspring were randomly assigned to an *ad libitum* diet of either standard Chow or HF, until the end of the study (P160).

Chow-fed Males UNP offspring, lighter than CONT offspring at weaning, put on more weight than the CONT group, catching up and surpassing them by P78, and becoming statistically heavier than CONT from P126. The UNL pups started off with body weights that were only slightly though significantly heavier than the UNPL group. However, they gained weight more rapidly than the UNPLs – the growth curves for the two groups became significantly different from P63 onward. The UNLs began to catch-up to body weights of the CONT group, and from P84 there was no longer a significant difference between the growth curves of these two groups.

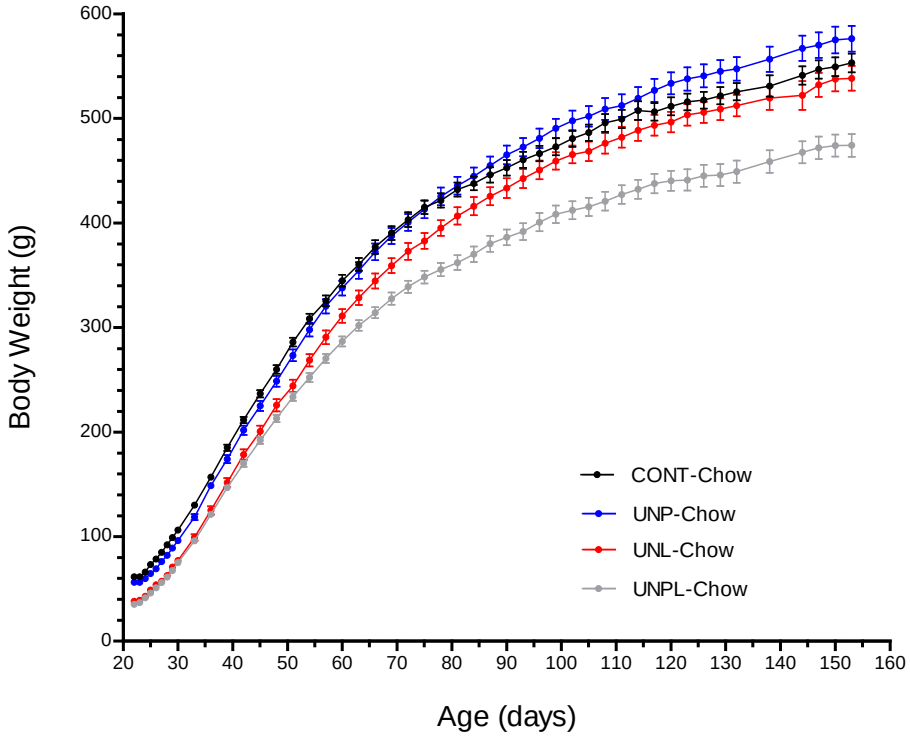


Figure 3.7: Post-weaning growth curves of chow-fed male offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL). Since all these offspring groups were raised on a post-weaning diet of Chow, their growth curves are reflective of maternal dietary history. Data are means ± SEM. *n* = 12-18 per group. Two Way repeated measures ANOVA with maternal diet and age as factors.

The UNPL group, lightest at weaning, made the least growth gain. A significant difference in body weight between them and the CONT group developed early in the postnatal period, i.e. from P26, and this continued to widen throughout the rest of the study. The UNPLs were also significantly lighter than the UNPs from P36 and the UNLs from P63. (Figure 3.7)

Chow-fed Females The female offspring exhibited a similar pattern of growth to the males, although they did not grow as big as the males.

The UNP pups exhibited catch-up growth, surpassing the CONTs from P72. However, the UNP growth curve was not significantly different from the CONT growth curve.

As with the males, the UNL females gained weight more rapidly than the UNPL group. Moreover the UNL growth curve began to approximate that of the CONT group, and there was no longer a significant difference between the CONT and UNL growth curves from P51 onward. The gap between the UNL and UNPL growth curves continued to expand throughout the post-weaning period.

The UNPL group had the slowest weight gain of all groups. The UNPL curve was significantly different from both the CONT and UNP growth curves for the entire post-weaning period, and it was different from the UNL growth curve from P117. (Figure 3.8)

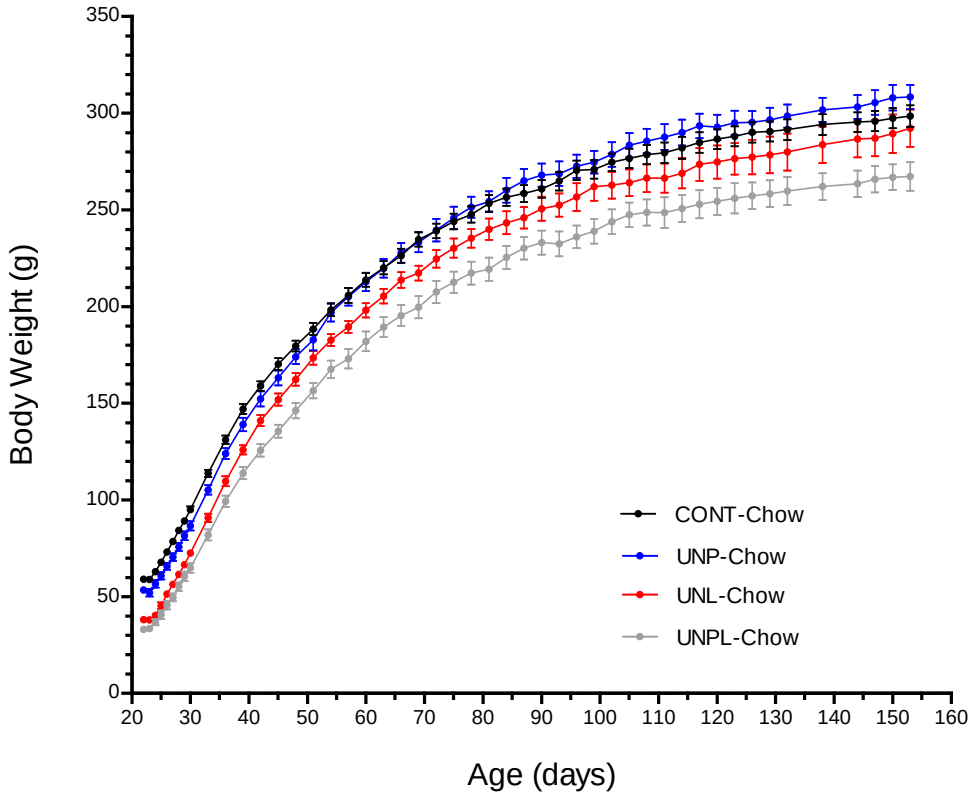


Figure 3.8: Post-weaning growth curves of chow-fed female offspring, of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL). Since these offspring groups were all raised on a post-weaning diet of Chow, their growth curves are reflective of maternal dietary history. Data are means ± SEM. *n* = 12-18 per group. Two Way repeated measures ANOVA, with maternal diet and age as factors.

3.3.9 High fat feeding: the post-weaning diet effect

A post-weaning HF diet always led to increased weight gain over Chow-fed siblings, in both males and females, regardless of maternal diet. However, this diet-induced obesity (DIO) in offspring was different between groups. (Two Way repeated measures ANOVA with post-weaning diet and age as factors. Significance set at $P<0.05$)

UNP offspring The UNP pups experienced catch-up growth after birth, but were still lighter than the CONT pups at weaning, in both males and females. A post-weaning HF diet led to an accelerated weight gain, so that UNP-HF offspring became significantly heavier then UNP-Chow offspring on P51, and their growth curves continued to widen thereafter, in both males and females. In males, however, UNP-HF offspring were never quite as heavy as the CONT-HF group. A significant difference emerged from P138 to the end of the study, when the CONT-HF growth curve was statistically elevated over that of the UNP-HF offspring. (Figure 3.9A)

The UNP female offspring exhibited a different growth trajectory compared to the UNP males. The UNP-HF females surpassed the CONT-HF females on Day 63 and remained heavier than them for the rest of the study. However, the difference between the growth curves of CONT-HF *versus* UNP-HF female offspring was not statistically significant. (Figure 3.9B)

UNL Offspring There was a significant post-weaning diet effect in both male and female UNL offspring ($P<0.05$). In males, UNL-HF offspring became significantly heavier than UNL-Chow offspring from P66, and in females from P57. The UNL-HF offspring, however, never caught up to the CONT-HF offspring in absolute body weight, in either males or females. Nevertheless, in females the growth curves between UNL-HF and CONT-HF tended to converge, and after P96 were no longer significantly different. By contrast, in the males a significant difference between CONT-HF and UNL-HF offspring emerged on P33, and the two growth curves continued to widen throughout the rest of the trial. (Figure 3.9, C&D)

UNPL Offspring Although the UNPL pups had the lightest mean bodyweight at both birth and weaning, they exhibited a significant post-weaning diet effect. UNPL-HF offspring were significantly heavier than UNPL-Chow offspring from P66 in males and P48 in females, with the growth curves of the two post-weaning dietary conditions continuing to wider for the rest of the study, in both genders. A post-weaning HF diet produced weight gain so that UNPL-HF offspring had similar growth curves to CONT-Chow offspring from P78 in males, and from P48 in females. (Figure 3.9, E&F)

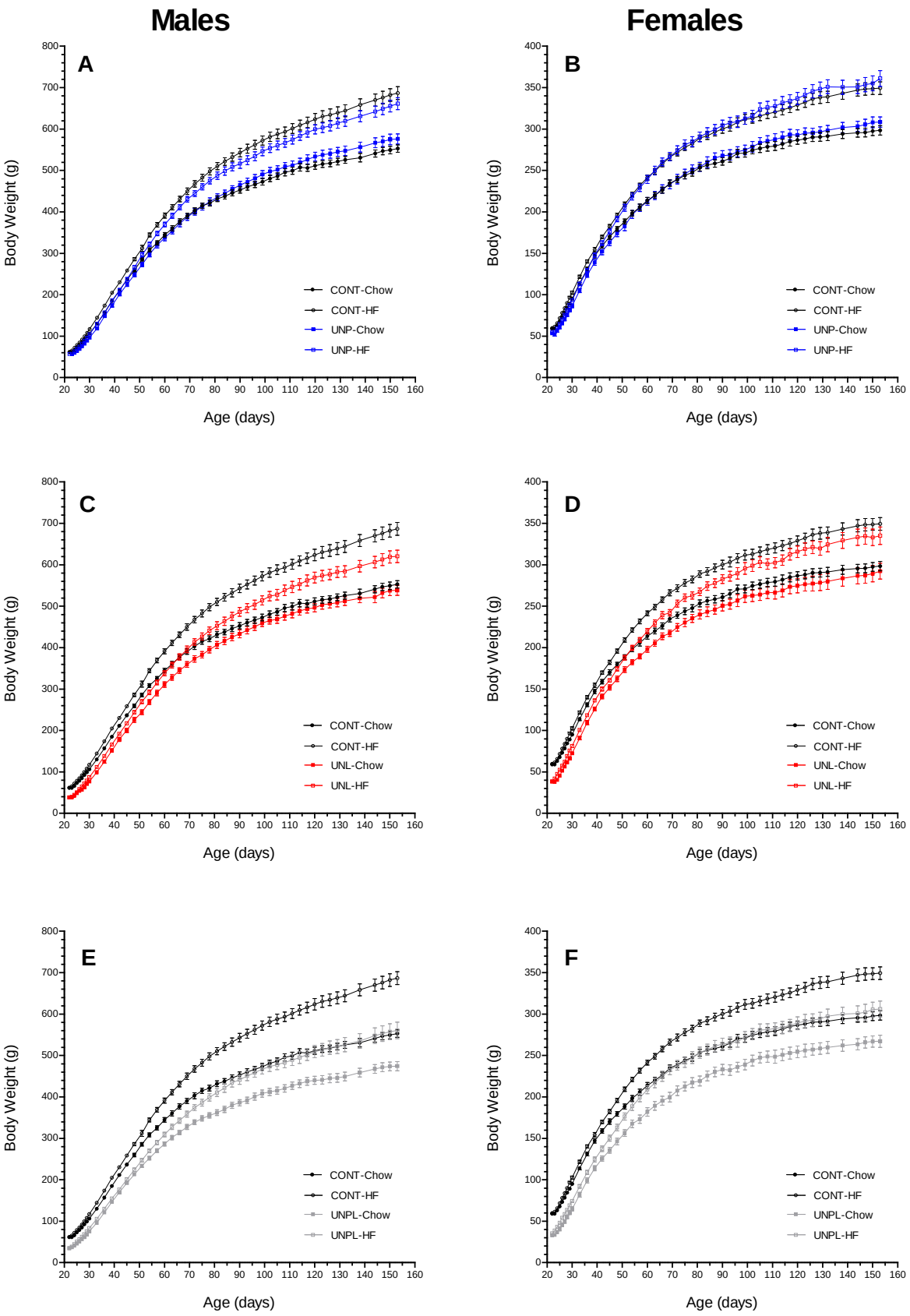


Figure 3.9: Post-weaning growth curves. (A) Male and (B) female offspring of chow-fed control dams (CONT) and offspring of dams fed 50% undernutrition throughout pregnancy (UNP), raised on chow or a high fat (HF) diet after weaning. (C) Male and (D) female offspring of CONT dams and offspring of dams fed 50% undernutrition throughout lactation (UNL), raised on chow or a high fat (HF) diet after weaning. (E) Male and (F) female offspring of CONT dams and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL), raised on chow or a high fat (HF) diet after weaning. Data are means ± SEM, *n* = 12-18 per group. *P*<0.05 for post-weaning diet effect (Two Way repeated measures ANOVA with post-weaning diet and age as factors).

3.3.10 Body composition

Animals underwent DEXA scanning at day P150, one week prior to completion of the trial.

UNP males exhibited a highly significant maternal diet effect, with increased body fat mass compared to all other groups ($P<0.001$). In the Chow-fed males, there was a 45% increase in adiposity in UNP animals over CONT (Figure 3.10A), whereas CONT and UNL and UNPL male offspring all had similar body fat percentages, in both the Chow-fed and HF-fed cohorts.

In female offspring there was a comparable result, though not as pronounced as in the males: UNP females again had the largest % body fat, and this was significantly increased compared to UNL and UNPL groups, but not against CONT (maternal diet effect, $P<0.001$, Figure 3.10B). However, in contrast to males, % body fat was reduced in UNL and UNPL female offspring compared to CONT offspring (25% and 24% reduction in UNL and UNPL females compared to CONT respectively), and this reached significance in the CONT *versus* UNL offspring.

There was a postnatal HF diet effect in all groups ($P<0.001$), equally so in both males and females (Figure 3.10, A&B).

Diet-induced obesity was assessed as the difference in mean % body fat between the HF-fed animals *versus* Chow-fed animals, within groups for each maternal dietary background. The UNPL animals approximated the CONT groups most closely, but there was no significant difference between any of the groups, in either males or females.

In fat: lean tissue ratio (grams body weight by DEXA), the UNP males were significantly different from CONT, UNL and UNPL offspring groups. In females the result was similar, with UNP fat: lean ratio significantly elevated over UNL and UNPL offspring, but not CONT ($P<0.001$, both genders). There was a significant post-weaning diet effect within all offspring groups, in both genders ($P<0.001$).

In actual weight of lean tissue (g), there was a maternal diet effect in males, in CONT offspring *versus* UNP and UNPL (but not UNL) offspring, and UNL *vs* UNPL offspring, $P<0.001$. There was an overall post-weaning diet effect ($P<0.001$) in males, seen within the CONT and UNP offspring groups only. There was no significant maternal diet effect seen in females, and although there was an overall significant post-weaning diet effect in female offspring ($P=0.006$), this was not significant within any one individual group.

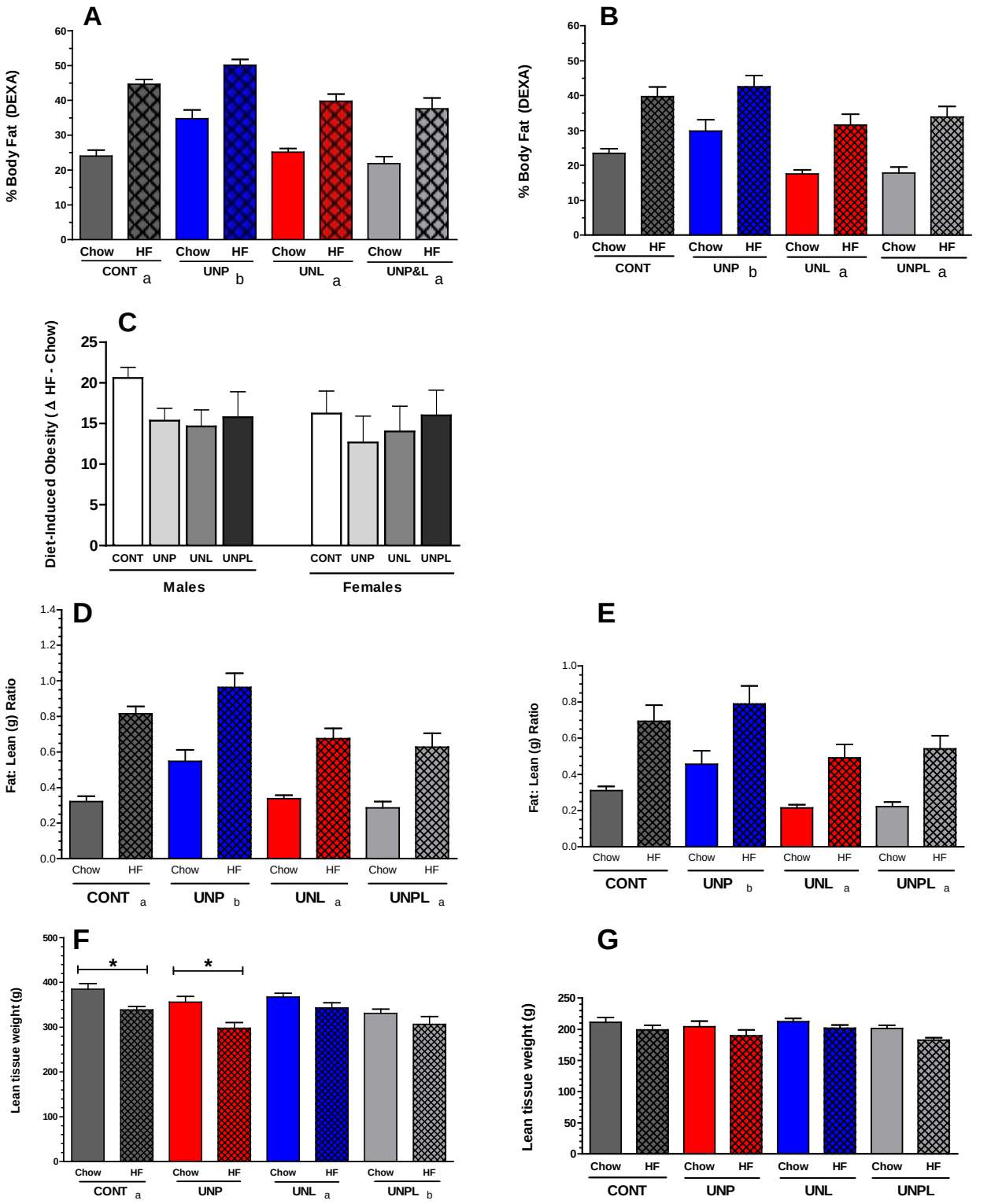


Figure 3.10: Body composition in male and female offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL), and raised on either chow or a high fat (HF) diet after weaning, at postnatal day 150. Adiposity (percent total body fat as quantified by DEXA scanning) in (A) male and (B) female offspring, $P<0.001$ for both maternal diet effect and postnatal diet effect. (C) Diet-induced obesity (% body fat: difference between means of HF-fed animals and Chow-fed animals for each maternal dietary group), NS in males and females. Fat: lean ratio in (D) male and (E) female offspring, $P<0.001$ for maternal diet and postnatal diet effect. Lean tissue weight in (F) male and (G) female offspring, $P=0.001$ for maternal diet and postnatal diet effect in males (*), $P=0.006$ for postnatal diet effect in females. Data are means ± SEM, n = 8-10 per group. Groups with different letters are significantly different from each other. (Two Way ANOVA with maternal diet and postnatal diet as factors).

3.3.11 Bone mineral content and bone mineral density

Overall, BMC was significantly higher in males compared to females for all maternal dietary groups (P<0.005 for effect of gender). In males, bone mineral content (BMC) was significantly higher in UNP males compared to all other groups, and was lower in UNPL males compared to CONT and UNL groups (P<0.001). In females, the UNP offspring again had the highest BMC, and the UNPL offspring had the lowest. BMC was significantly higher in UNP *versus* UNL and UNPL offspring, and also in CONT *versus* UNPL offspring (P<0.001). There was a pronounced postnatal diet effect, Chow < HF, for BMC in all groups, and in both genders (P<0.001, Table 3.2).

Table 3.2: **Bone mineral content (BMC) and bone mineral density (BMD)**

Maternal Diet	Postnatal Diet	Males		Females	
		Bone Mineral Content	Bone Mineral Density	Bone Mineral Content	Bone Mineral Density
CONT	Chow	13.1 ± 0.3 [b]	0.173 ± 0.002	7.9 ± 0.2 [d]	0.159 ± 0.001
	HF	16.9 ± 0.4	0.184 ± 0.002	10.2 ± 0.3	0.170 ± 0.002
UNP	Chow	15.2 ± 0.4 [acd]	0.180 ± 0.002 [cd]	8.5 ± 0.2 [cd]	0.165 ± 0.003 [cd]
	HF	17.0 ± 0.5	0.185 ± 0.002	10.5 ± 0.5	0.165 ± 0.003
UNL	Chow	12.7 ± 0.4 [b]	0.173 ± 0.002 [b]	7.7 ± 0.2 [b]	0.156 ± 0.002 [b]
	HF	16.1 ± 0.6	0.179 ± 0.002	9.1 ± 0.3	0.165 ± 0.002
UNPL	Chow	11.3 ± 0.5 [b]	0.172 ± 0.004 [b]	7.1 ± 0.3 [ab]	0.154 ± 0.003 [b]
	HF	14.2 ± 0.6	0.178 ± 0.002	8.9 ± 0.4	0.166 ± 0.001

Data are means ± SEM; n = 8-10 per group.
CONT = control offspring; UNP = offspring of dams fed 50% undernutrition throughout pregnancy; UNL = offspring of dams fed 50% throughout lactation; UNPL = offspring of dams fed 50% undernutrition during pregnancy and lactation; chow = chow-fed; HF = high fat-fed.
a/b/c/d denote significant differences in maternal diet effect: a = different from CONT; b = different from UNP; c = different from UNL; d = different from UNPL.

Bone mineral density (BMD) showed a consistent gender effect, being higher in males than in females, in all groups (P<0.001). BMD was slightly but significantly higher in UNP animals compared to UNL and UNPL offspring, in both genders (P<0.05 for each comparison, see Table 3.2). In males, a postnatal HF diet increased BMD in all groups, but this was significant only within the CONT animals. By contrast, in females, the postnatal diet effect was significant within CONT, UNL and UNPL groups, with no difference at all within the UNP female group.

3.3.12 Tissue Weights

The UNP offspring and the UNPL offspring tended to exhibit the most changes in relative organ and tissue weights in comparison to Controls (see Table 3.3).

Retroperitoneal Fat Pads There was a significant maternal diet effect in retroperitoneal fat pad weight expressed relative to body weight, in both males and females ($P<0.001$).
In males, retroperitoneal fat pads (expressed as a percentage relative to body weight = %BW) were significantly increased in the UNP group compared to CONT and UNPL offspring and tended towards significance in the UNP *versus* UNL group ($P=0.06$). The CONT males also had significantly heavier retroperitoneal fat pads (%BW) than UNPL males, which were the lightest of the male groups.
In females, the UNP animals had significantly larger retroperitoneal fat pads (%BW), compared to all other groups. Retroperitoneal fat pad weights (%BW) were significantly reduced in both UNL and UNPL female offspring compared to CONT offspring.
There was a highly significant postnatal diet effect on retroperitoneal fat pad weight relative to body weight, in all groups, in both males and females ($P<0.001$).

Liver Relative liver weights (%BW) were significantly increased in male Chow-fed UNP offspring compared to CONT and UNL animals, although there was no difference between any of the HF-fed males. Relative liver weights were not different between any of the female groups. There was a highly significant postnatal diet effect on relative weight of liver, in all groups, in both males and females ($P<0.001$).

Spleen Relative spleen weights in males were significantly reduced in UNP offspring compared to UNL and UNPL animals. There was a similar trend in females, with UNP relative spleen weight reduced against other groups, but this did not quite reach significance ($P=0.063$).
Postnatal diet reduced relative spleen weight in CONT, UNL and UNPL males ($P<0.001$), but not UNP males. There was no significant postnatal diet effect in any of the female offspring.

Kidneys Relative weight of kidneys in males was significantly increased in UNPL offspring compared to CONT animals. Kidney weights (%BW) were not different in females between any of the groups (Table 3.3). Postnatal HF feeding tended to reduce relative kidney weight: this was significant within CONT and UNP males, and within UNL females.

Adrenals There were no differences in relative adrenal weights between any of the male offspring. In females, relative adrenal weights in UNPL animals were increased compared to UNP offspring. Postnatal HF feeding reduced relative adrenal weight within CONT and UNP males (P=0.024), and within all female groups (P<0.001).

Heart Male offspring showed a highly significant overall maternal diet effect in heart weight relative to total body weight (P<0.001). UNP males showed a reduction in relative heart size (%BW) against UNL and UNPL offspring, with CONT also reduced *versus* UNPL animals. In females, UNP offspring had reduced relative heart weights when compared to the UNL group. A HF postnatal diet reduced relative heart weight in CONT and UNP males, but had no significant effect within any of the female offspring groups.

All statistical analysis: Two Way ANOVA, with maternal diet and postnatal diet as factors.

Table 3.3: **Tissue weights (expressed relative to total body weights), in adult male & female offspring**

Males	Retro Fat Pads (%BW)	Liver (%BW)	Spleen (%BW)	Kidneys (%BW)	Adrenals (%BW)	Heart (%BW)
CONT-Chow	2.31±0.17 [d]	2.84±0.05	0.21±0.01	0.61±0.02	0.011±0.001	0.27±0.01
CONT-HF	4.20±0.22	2.48±0.07	0.18±0.01	0.53±0.01	0.009±0.001	0.24±0.01
UNP-Chow	2.79±0.21 [ad]	3.24±0.16 [ac] *	0.19± 0.01	0.63±0.02	0.012±0.001	0.24±0.01
UNP-HF	4.35±0.21	2.45±0.07	0.18±0.01	0.53±0.02	0.010±0.001	0.23±0.01
UNL-Chow	2.32±0.08	2.85±0.08	0.23± 0.01 [b]	0.61±0.01	0.011±0.001	0.28±0.01 [b]
UNL-HF	3.66±0.23	2.38±0.05	0.20±0.01	0.57±0.01	0.010±0.001	0.26±0.01
UNPL-Chow	1.99±0.16	3.12±0.13	0.23± 0.01 [b]	0.64±0.01 [a]	0.011±0.001	0.28±0.01 [ab]
UNPL-HF	3.31±0.23	2.64±0.07	0.20±0.01	0.60±0.01	0.011±0.001	0.27±0.01

Females	Retro Fat Pads (%BW)	Liver (%BW)	Spleen (%BW)	Kidneys (%BW)	Adrenals (%BW)	Heart (%BW)
CONT-Chow	1.45±0.08	2.91±0.06	0.26±0.01	0.68±0.01	0.026±0.001	0.33±0.01
CONT-HF	2.10±0.11	2.38±0.07	0.30±0.02	0.64±0.01	0.022±0.001	0.31±0.01
UNP-Chow	1.70±0.11 [acd]	2.79±0.11	0.26±0.01	0.65±0.02	0.026±0.001	0.32±0.01
UNP-HF	2.34±0.11	2.41±0.06	0.25±0.01	0.62±0.02	0.020±0.001	0.30±0.01
UNL-Chow	1.07±0.09 [ab]	2.76±0.04	0.28±0.01	0.67±0.01	0.029±0.001	0.35±0.01 [b]
UNL-HF	1.78±0.17	2.36±0.05	0.27±0.01	0.62±0.02	0.021±0.001	0.32±0.01
UNPL-Chow	1.10±0.08 [ab]	2.94±0.08	0.31±0.03	0.66±0.01	0.028±0.001 [b]	0.34±0.01
UNPL-HF	1.90±0.15	2.56±0.06	0.29±0.02	0.65±0.02	0.024±0.001	0.32±0.01

Data are means ± SEM, *n* = 12-18 per group.

CONT = control offspring; UNP = offspring of dams fed 50% undernutrition throughout pregnancy; UNL = offspring of dams fed 50% throughout lactation; UNPL = offspring of dams fed 50% undernutrition during pregnancy and lactation; chow = chow-fed; HF = high fat-fed.

a/b/c/d denote significant differences in maternal diet effect: a = different from CONT; b = different from UNP; c = different from UNL; d = different from UNPL.

Note: differences simply within Chow-fed or within HF-fed cohorts are not superscripted on this table, with one exception: in the Liver (%BW) column, marked with an asterisk (*), where differences are between Chow-fed male animals only.

3.3.13 Offspring glucose, insulin and leptin

Fasting plasma glucose NS difference between any of the groups. (Table 3.4)

Table 3.4: **Fasting plasma glucose (mmol/L) in adult offspring (day P160)**

	CONT		UNP		UNL		UNPL	
	Chow	HF	Chow	HF	Chow	HF	Chow	HF
Males	5.6 ± 0.6	6.0 ± 1.0	5.7 ± 1.3	6.1 ± 0.6	5.6 ± 0.6	5.9 ± 0.8	6.2 ± 0.7	6.0 ± 0.5
Females	5.7 ± 0.7	5.6 ± 0.5	5.4 ± 0.8	5.5 ± 0.6	5.4 ± 0.7	5.5 ± 0.8	5.3 ± 0.6	5.5 ± 0.5

Data are means ± SEM, *n* = 12-18 per group
CONT = control offspring; UNP = offspring of dams fed 50% undernutrition throughout pregnancy; UNL = offspring of dams fed 50% throughout lactation; UNPL = offspring of dams fed 50% undernutrition during pregnancy and lactation; chow = chow-fed; HF = high fat-fed.

Fasting plasma insulin Maternal dietary background had no significant effect across either male or female groups. However, a postnatal HF diet significantly increased plasma insulin within all groups, in both male and female offspring ($P<0.001$). (Figure 3.11 A&B)

Fasting plasma leptin In parallel with increased total body fat mass, fasting plasma leptin concentrations were significantly increased in UNP males and females ($P<0.001$, for both sexes). In males, UNP leptin levels were significantly elevated against UNL and UNPL offspring, whereas UNPL offspring had the lowest leptin levels, reaching significance *versus* the CONT group. In the female cohorts, UNP leptin concentrations were significantly elevated against all three other groups. A postnatal HF diet produced a significant increase in leptin levels in all groups, in both male and female offspring ($P<0.001$). (Figure 3.11 C&D)

Insulin:leptin ratios Plasma insulin:leptin ratios were significantly influenced by maternal dietary background, in both male and female offspring ($P<0.001$ and $P<0.002$, respectively). In males, the UNP offspring had reduced insulin:leptin ratios compared to all other groups. Ratios in UNL and UNPL males were similar but both elevated *versus* CONT. In females, insulin:leptin ratios were increased in UNL and UNPL offspring compared to UNP animals, but were not different between CONT and UNP groups. (Figure 3.11)

A postnatal diet effect was evident within CONT and UNPL males, where HF feeding decreased the insulin:leptin ratio significantly, a trend that was also present within the UNL males

(*P*=0.067), but which was noticeably absent within the UNP group (*P*=0.96). There was no postnatal diet effect within the female offspring. (Figure 3.11 E&F)

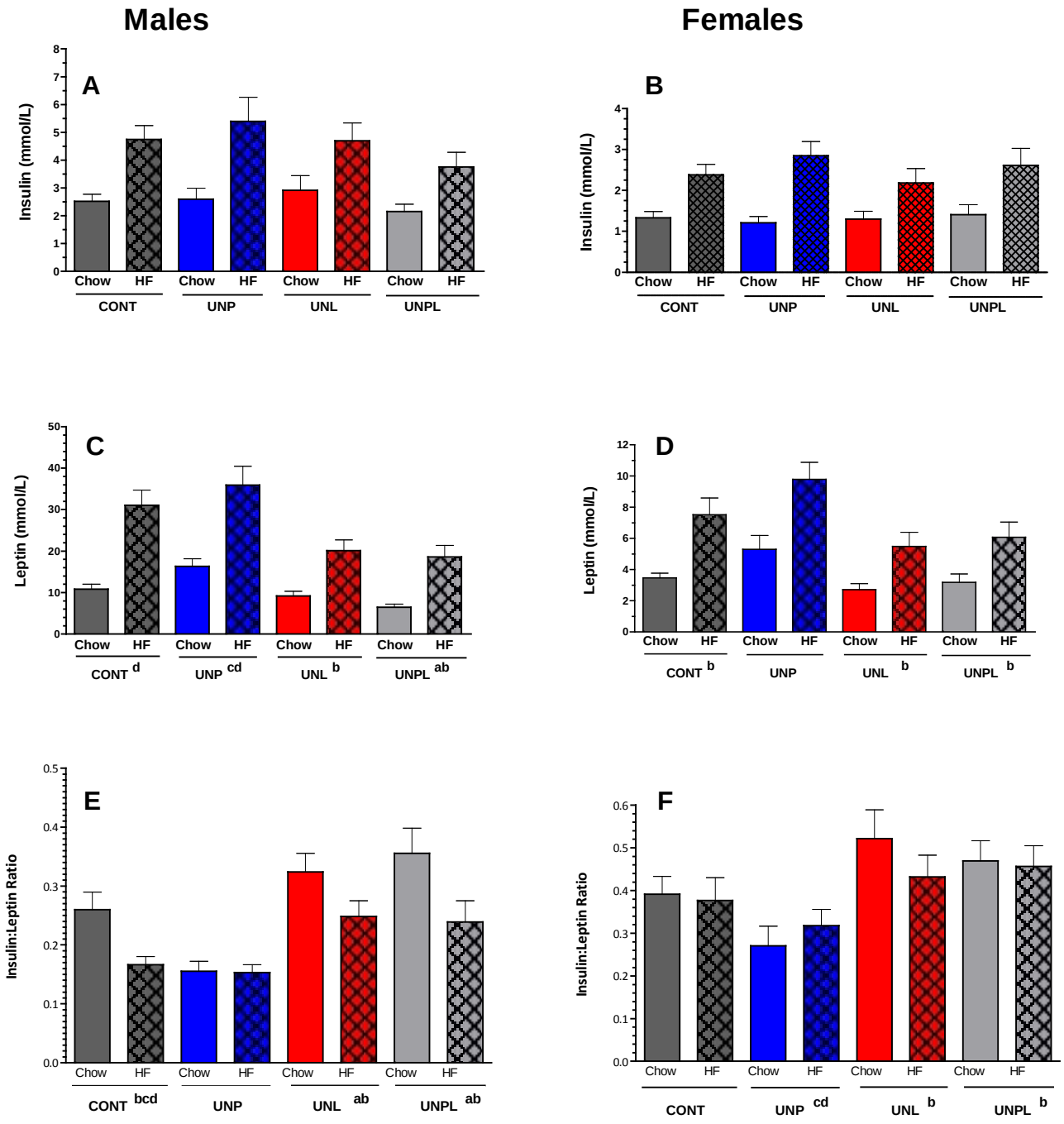


Figure 3.11: Fasting insulin (A and B) and leptin concentrations (C and D) and insulin:leptin ratios (E and F) in chow- or high fat (HF)-fed offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL), at postnatal day 160. (A and B): *P*<0.001 for post-weaning diet, effect in both males and females. (C and D): *P*<0.001 for maternal diet effect and post-weaning diet effect, in both males and females. (E and F): *P*<0.001 for maternal diet effect and post-weaning diet effect in males; *P*<0.002 for maternal diet effect in females. Letters denote a significant maternal diet effect between groups: a = different from CONT, b = different from UNP, c = different from UNL, d = different from UNPL . (Two Way ANOVA with maternal diet and postnatal diet as factors). Data are means ± SEM, *n* = 8-12 per group.

3.3.14 Post-weaning food consumption and caloric intake

Food consumption (g) In Chow-fed males, for unadjusted food intake (grams consumed) over the course of the trial, there was no significant difference between CONT *vs* UNP *vs* UNL groups. However, UNPL-Chow males consumed significantly less than CONT and UNP Chow-fed males. In the HF-fed males there was no significant difference in food consumption between any of the groups (data not shown).

In females, the Chow-fed animals all ate very similar weights of food per day. The only significant difference seen was CONT *vs* UNPL, but this was present only during the first post-weaning week, and thereafter (from P30) all groups were NS against each other. A very similar pattern was seen in the HF-fed females: there was a significant difference between the CONT and UNPL food consumption curves during the first seven days, but thereafter there was no difference in consumption between groups (data not shown).

Caloric intake Amongst the Chow-fed animals, relative caloric intake (kcals consumed /g body weight per day) was significantly increased in UNL and UNPL offspring, in both males and females, but only from weaning until approximately P30, when daily caloric intake returned to match that of CONT and UNP offspring for the rest of the trial (Figure 3.12A, males only, female data similar).

In the HF-fed animals the pattern was very similar, in both males and females: UNL and UNPL offspring had a significantly increased energy intake over CONT and UNP offspring, but for no more than the first week after weaning (data not shown, but see female CONT-HF and UNP-HF embedded within Figure 3.12B). When energy intake curves are compared between Chow-fed and HF-fed cohorts, there is no significant difference between any of the groups beyond the first week after weaning, in either males or females (Figure 3.12B, CONT and UNP females only, male data similar).

There were no differences in food consumed (grams) or caloric intake (kcal/g) between CONT and UNP offspring (Figure 3.12B, caloric intake, females only, but male data similar).

Gender differences Female offspring of all groups consistently had a higher energy intake than males from day P64, irrespective of whether they were fed a post-weaning diet of Chow or HF (Figure 3.13, Chow-fed males and females only, HF-fed groups had similar data).

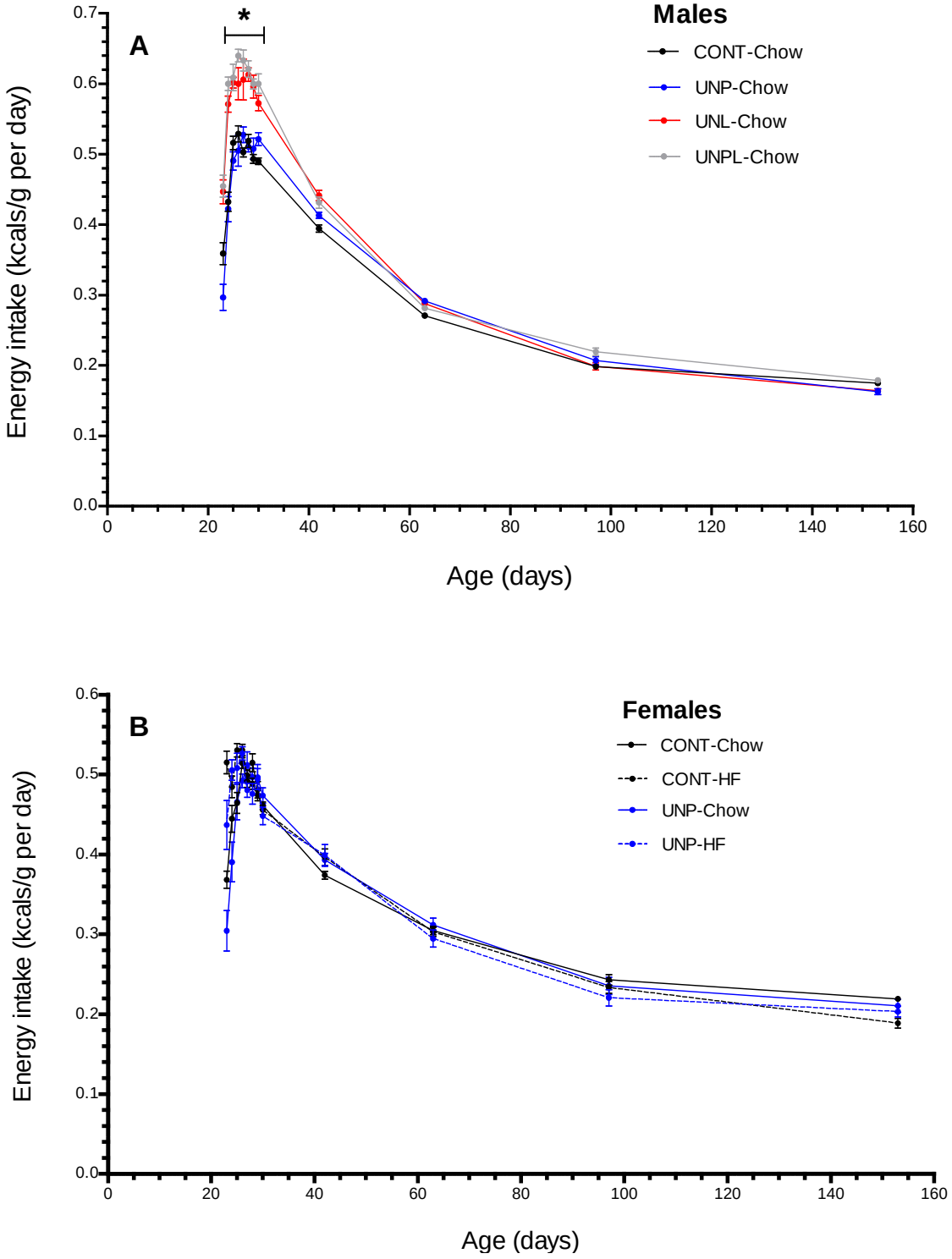


Figure 3.12: Offspring post-weaning energy intake (kcal/g body weight per day), P22-P160, in (A) male chow-fed offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL): $P<0.05$ UNL and UNPL *versus* CONT and UNP until age P30 only (*); and in (B) female chow- and high fat (HF)-fed offspring of chow-fed control dams (CONT) *versus* offspring of dams fed 50% undernutrition throughout pregnancy (UNP): NS difference between groups. Data are means ± SEM. *n* = 12-18 animals per group. Two Way Repeated Measures ANOVA, with maternal diet and postnatal diet as factors.

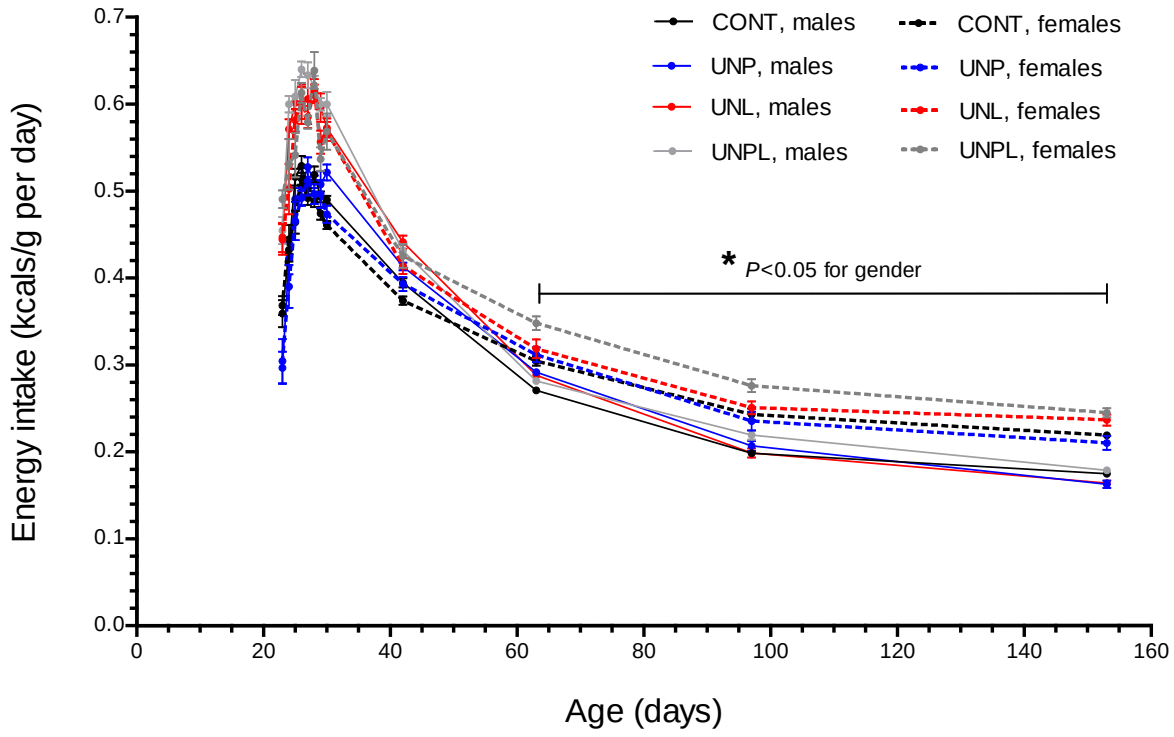


Figure 3.13: Offspring post-weaning energy intake (kcal/g body weight per day), P22-P160, in chow-fed male and female offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL): Overall energy intake curves, days P22 - 153, males *versus* females are not statistically different; but for days P64 – 153 only, males *versus* females, $P<0.05$ for gender (*). Data are means ± SEM. n = 12-18 animals per group. Two Way repeated measures ANOVA, with gender and age as factors.

3.3.15 Onset of Puberty

CONT offspring Age of onset of puberty in the offspring of CONT dams was similar to that reported previously (322). CONT females entered puberty at an earlier age than CONT males, irrespective of postnatal diet ($P<0.001$). A post-weaning diet of HF alone advanced the age of pubertal onset in both female and male offspring of CONT dams ($P=0.001$, Two Way ANOVA, with sex and postnatal diet as factors).

Female offspring Irrespective of postnatal diet, a maternal diet of 50% undernutrition resulted in early onset of puberty ($P=0.001$). Maternal undernutrition during pregnancy and/or lactation significantly brought the age of pubertal onset down, to a similar degree in all three UN cohorts, whether UNP, UNL or UNPL (Figure 3.14A). The addition of a HF postnatal diet further advanced the age of puberty across all groups ($P<0.001$), (Figure 3.14 C, E&G, and Table 3.5).

Male Offspring As with the females, maternal UN resulted in earlier onset of puberty in male offspring, in all groups, regardless of the window of maternal dietary restriction ($P<0.001$). All Chow-fed UN groups experienced advanced pubertal onset compared to CONT-Chow, and the UNPL-Chow males were also significantly advanced *versus* the UNP-Chow group (Figure 3.14B). The addition of a HF post-weaning diet further advanced onset of puberty in all groups; this difference was significant within the CONT and UNL cohorts ($P<0.05$), but not within the UNP and UNPL males (Figure 3.14 D, F&H, and Table 3.5).

Weight on attaining puberty, female and male offspring In both females and males there was a highly significant effect of maternal diet on offspring weight at pubertal onset. A maternal diet of restricted nutrition, irrespective of whether the window of UN was pregnancy and/or lactation, led to offspring entering puberty at significantly lower body weights compared to CONT offspring ($P<0.001$, for both females and males; Two Way ANOVA with maternal diet and postnatal diet as factors, for each gender). There was no postnatal diet effect, in either female or male offspring, on body weight at pubertal onset (Table 3.5).

Table 3.5: **Age and weight at puberty**

Group	Females		Males	
	Pubertal Age (days)	**Pubertal Weight (g)**	**Pubertal Age (days)**	**Pubertal Weight (g)**
CONT-Chow	34.6 ± 0.5	121.50 ± 3.7	37.4 ± 0.4	170.25 ± 4.1
CONT-HF	32.8 ± 0.4	116.7 ± 6.3	35.4 ± 0.6	173.6 ± 6.7
UNP-Chow	33.3 ± 0.3 [a]	111.6 ± 5.6 [a]	35.6 ± 0.7 [a]	143.8 ± 5.4 [a]
UNP-HF	31.8 ± 0.2	105.9 ± 2.4	35.2 ± 0.5	148.6 ± 4.3
UNL-Chow	33.5 ± 0.3 [a]	94.6 ± 3.8 [a]	34.8 ± 0.5 [a]	119.7 ± 6.2 [a]
UNL-HF	31.8 ± 0.3	92.1 ± 2.8	32.7 ± 0.5	109.6 ± 4.4
UNPL-Chow	33.3 ± 0.4 [a]	84.9 ± 3.6 [a]	34.1 ± 0.4 [a,b]	102.8 ± 3.4 [a]
UNPL-HF	30.7 ± 0.3	77.9 ± 3.3	33.6 ± 0.5	112.8 ± 3.6

Data are means ± SEM, *n* = 12-18 per group.

CONT = control offspring; UNP = offspring of dams fed 50% undernutrition throughout pregnancy; UNL = offspring of dams fed 50% throughout lactation; UNPL = offspring of dams fed 50% undernutrition during pregnancy and lactation; chow = chow-fed; HF = high fat-fed.

Pubertal age: $P<0.001$ for maternal diet effect and post-weaning diet effect, in both females and males.

Pubertal weight: $P<0.001$ for maternal diet effect, in both females and males.

a/b denote significant differences in maternal diet effect: a = different from CONT-Chow; b = different from UNP-Chow.

Two Way ANOVA using Fisher PLSD method, with maternal diet and postnatal diet as factors.

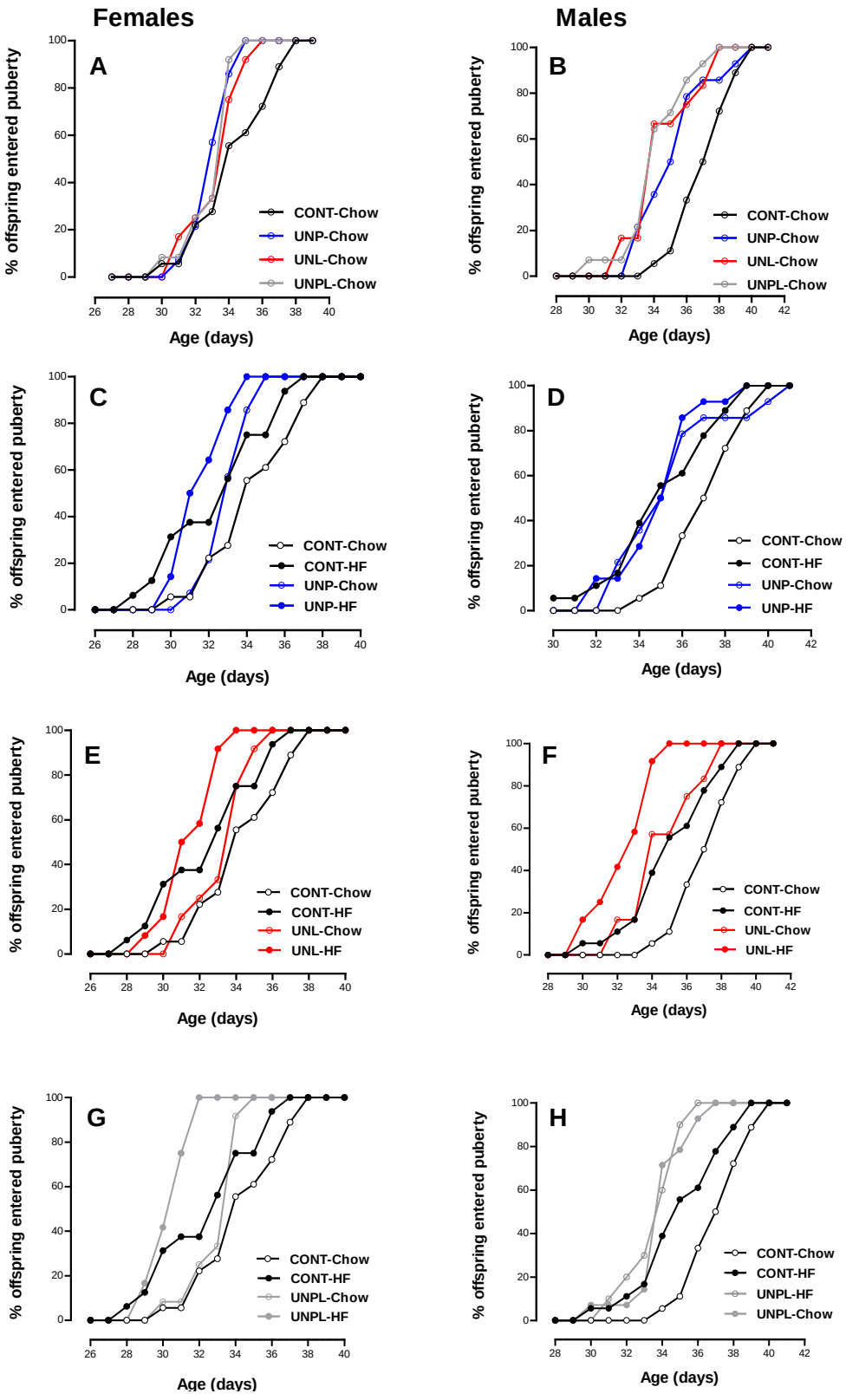


Figure 3.14: Age of pubertal onset (% of offspring entering puberty over time). (A) Female and (B) male chow-fed offspring of chow-fed control dams (CONT), offspring of dams fed 50% undernutrition throughout pregnancy (UNP), offspring of dams fed 50% undernutrition throughout lactation (UNL), and offspring of dams fed 50% undernutrition during pregnancy and lactation (UNPL): $P<0.001$ for maternal diet effect in both males and females. (C) Female and (D) male offspring of CONT and UNP dams, fed either chow or a HF diet after weaning; (E) female and (F) male offspring of CONT and UNL dams, fed either chow or a HF diet after weaning; (G) female and (H) male offspring of CONT and UNPL dams, fed either chow or a HF diet after weaning: $P<0.001$ for maternal diet effect and postnatal diet effect (C)-(H). Data are percentage of total pups entered puberty, n = 12-18 pups per group. (Three Way ANOVA with maternal diet, postnatal diet and gender as factors.)

3.4 Discussion

Maternal undernutrition during pregnancy is well known to result in increased adiposity and related metabolic disease risk factors in offspring (114, 296, 323). The present study highlights that the timing and duration of the period of undernutrition is critical to the development of the obese/metabolic phenotype. Extension of the period of undernutrition into lactation can partially prevent the adverse consequences associated with gestational undernutrition and, moreover, these effects are gender dependent.

Maternal undernutrition during pregnancy produced the severest effects on offspring phenotype. The UNP pups were intrauterine growth retarded (IUGR), born significantly lighter than the CONT pups, and shorter in both NA and NT lengths. The return of UNP dams to *ad libitum* Chow during lactation, provided the IUGR UNP pups with an abundant postnatal food supply, resulting in rapid catch-up growth. The spike in maternal energy intake that occurred immediately following parturition when UNP dams were again permitted *ad libitum* nutrition likely contributed to this catch-up phenomenon. This increase in offspring body weight was due to increased adiposity, as revealed by DEXA body composition scanning and by weight of retroperitoneal fat pads relative to body weight. Increased adiposity was paralleled by elevated plasma leptin levels in adult UNP females and a reduced insulin:leptin ratio in males, indicative of leptin resistance. Interestingly, moderate undernutrition during pregnancy alone did not have any significant effect on fasting plasma insulin or glucose concentrations.

Thus, as shown before (296, 324), in the presence of abundant neonatal nutrition, growth-restricted pups born to undernourished mothers demonstrate rapid catch-up growth, such that their body weights match or exceed those of controls early in postnatal life. This situation represents ‘mismatch’, where the *in utero* nutritional environment does not match the postnatal nutritional environment. It is hypothesised that such mismatch may contribute to the metabolic syndrome or other conditions of ill health, which manifest much later in adult life. Thus prevention of catch-up growth has been debated as an avenue to ameliorate the consequences of early life growth restriction (325).

Male offspring of dams undernourished during lactation alone had similar percentage body fat and fasting plasma insulin and leptin concentrations to those of Controls. In contrast, in UNL female offspring there was a significant reduction in percentage fat mass as compared to both CONT and UNP groups. This reduction in fat mass was not coupled with changes in plasma insulin or leptin compared to CONT. Moreover, the insulin:leptin ratio was elevated in male UNL offspring *versus* Controls, but not in females. This suggests that prevention of catch-up

growth in females in the pre-weaning period, *independent* of level of maternal nutrition during pregnancy, can confer lasting beneficial effects on body composition.

The UNPL pups exhibited the least weight gain during lactation, and remained small throughout the study. In both male and female offspring, percentage body fat mass and plasma insulin concentrations were similar to CONT. There was significant reduction in plasma leptin in male offspring, but in females leptin was similar to Controls. The insulin:leptin ratio was elevated in UNPL male offspring *versus* CONT and UNP groups, but only *versus* UNP offspring in females, suggestive of a leptin sensitivity equal to or increased over the Control animals.

These data show that different windows of maternal undernutrition have differential impacts on offspring phenotype. Undernutrition during the earlier window of gestational development is more deleterious, particularly when followed by catch-up growth due to a restored plane of nutrition. Undernutrition that followed normal *in utero* development did not have as severe effects, and may result in improved outcomes, especially for females. When undernutrition was continued through both windows of development (an example of 'match'), although animals remained small, they had metabolic profiles similar to Controls, with improved leptin sensitivity in males. Thus, limitation of post-natal nutrition has been proposed for IUGR babies in humans. However, these studies in rodents cannot be directly applied to humans who are born at a later stage of development than rats.

Tissues / organs

With the exception of retroperitoneal fat pad mass, the effects of moderate maternal undernutrition on tissue weights were relatively slight. Fat pad mass was paralleled by the DEXA assessment of percentage adiposity. Both reflect weight gain due to increased fat deposition, rather than growth of lean tissue. Actual lean tissue mass differed little between groups, with only the lightest group, the UNPL offspring, showing a difference from Controls, and then only in males.

Maternal undernutrition has been shown to result in a reduction in kidney size and nephron number but the lack of effect on renal mass in the present study most likely relates to a less severe level of undernutrition to that reported previously (326, 327). Overall, there are more changes in relative organ weights in the UNP offspring than in the other groups, which may reflect the degree of developmental disturbance that is possible through undernutrition nutrition during pregnancy followed by neonatal catch-up growth.

3.4.1 Postnatal diet effects

Post-Weaning Growth & Weight Gain A postnatal HF diet universally caused increased weight gain over Chow-fed animals, within all groups, and across both sexes. Similarly, there was a HF effect on body length, an increase in both NA and NT lengths, which was significant in almost all groups, in both genders. ($P<0.05$, Two Way Repeated Measures ANOVA, with Group *versus* Age as factors.)

Adiposity Postnatal HF feeding universally produced a significant increase in adiposity across all groups, in both males and females (% body fat, DEXA). ($P<0.001$, Two Way ANOVA with Maternal Diet and Postnatal Diet as factors.) This DEXA data was matched by the retroperitoneal fat pad data collected at cull – both in raw weight of retro fat pad and as %BW, significant within all groups, both males and females. The two sets of data were highly correlated across groups.

Gender differences in response to a postnatal HF diet The growth curves of UNP males and females both exhibited a classic postnatal diet effect, with the gap between Chow- *versus* HF-fed animals continuing to widen with time. However, they differed in their comparison with Controls. The UNP-HF males were never as heavy as the CONT-HF males. By contrast, the UNP females surpassed the CONT-HF females on day P63. The UNP animals were the only group where postnatal diet produced such a clear distinction between genders. It appears that male UNP offspring tolerated a post-weaning HF diet less well than either the CONT-HF males or the UNP-HF females, or they may have reached a ceiling in fat deposition.

Energy intake The postnatal diet effect was not due to difference in energy intake: both Chow- and HF-fed groups consumed equivalent kcals per gram of body weight. Thus, the group phenotypic differences in growth and body weight were achieved by differential utilisation and storage of equivalent caloric intake from the two diets. A post-weaning diet consisting largely of fat induced an alteration of how nutrients were metabolised and distributed within the rodent body (304, 328, 329). The mechanisms are most likely central, but also involve alterations in adipoinsular axial function.

Insulin, leptin and insulin:leptin ratios A post-weaning HF diet significantly elevated both plasma insulin and plasma leptin concentrations, in all treatment groups and in both genders. As a result the insulin:leptin ratio was almost always reduced in HF fed offspring, since plasma leptin typically rose more than insulin. This was particularly seen in males, reflective of the

susceptibility of males in general to deposit more fat than females, with concomitant elevated plasma leptin levels. However, in the UNP condition the insulin:leptin ratio was depressed in Chow-fed as well as HF-fed offspring, in both genders. These data signify the extent of raised leptin concentrations within the UNP cohorts, in parallel with their increased adiposity. By contrast, insulin:leptin ratios are raised in UNL and UNPL groups (significantly different against both CONT and UNP groups in males, and against UNPs alone in females), suggestive of improved leptin suppression of insulin mediated adipogenesis in these less adipose cohorts.
Male insulin:leptin ratios exhibited a wider range between groups than did females. This suggests that females were more tolerant than males to postnatal HF challenge, while males exhibited a different response with greater leptin resistance and impaired adipoinsular feedback.

3.4.2 Comparing males *versus* females

Size at birth Males were slightly heavier and slightly longer at birth than females, in all groups. However, these differences only reached significance in the UNP and UNPL groups – both of which experienced maternal undernutrition during gestation. This suggests that, in maternal undernutrition during gestation, there was an exaggeration of the normal tendency for male pups to be larger than females. It appears there may be a tendency, in states of nutritional deprivation, for male pups *in utero* to gain weight more readily than female pups (and perhaps at their expense).

Energy Intake There were no differences in energy intake (kcals/g body weight) between groups, in either the Chow or HF post-weaning dietary states, in either males or females. However, there may be a difference between the sexes. Energy intake curves were similar for both sexes in the early period of elevated intake from days P22-P42. Thereafter, as energy intake declined with age, the female curves were consistently above the male levels of caloric intake. A Two Way Repeated Measures ANOVA with gender and age as factors demonstrated a significant gender effect (females > males, $P<0.05$) for energy intake across days P64-P153 (see Figure 3.13). This pattern was seen in both the Chow-fed and HF-fed offspring, females *versus* males. The higher energy intake in females may have to do with thermoregulation and body size. Female rats are smaller than males, with a greater body surface area to mass ratio, thus they lose heat more readily. Females would need to expend more energy than males on maintaining body temperature.

% Body Fat Interestingly, adiposity (percentage body fat of total weight) in Chow-fed Control animals was similar in males *versus* females (% body fat: males, 24% ± 1.7; females

23.5% ± 1.3; DEXA data, P150). In contrast to this norm, offspring of dams that experienced dietary restriction were less gender equal in body composition, with males tending to greater adiposity over females. This tendency only reached significance, males > females, in the offspring of the UNL group – but interestingly, in both the Chow and HF post-weaning dietary states. This is further evidence that 50%UN during lactation is protective against future adiposity in a gender specific manner.

Moreover there was a gender difference in the degree of adiposity between groups. The male tendency to adiposity in conditions of maternal dietary restriction was relatively uniform across groups, with only the obese UNP males being different from Controls. In the females, however, adiposity became more divergent: in females the UNP offspring were not statistically different from Controls, but the UNL offspring had significantly less adiposity compared to CONT ($P<0.05$). Again, it was the restraint on neonatal catch-up growth that led to less adiposity, and this was gender specific, being significant only in females. Of note, it is also independent of the level of maternal nutrition during gestation.

Liver Liver size also exhibited a gender effect. Maternal undernutrition during pregnancy affected liver size much more in males than in females. In males the UNP group had the largest livers, both in raw weight and also as %BW (maternal diet effect, $P<0.001$ and $P<0.006$ respectively); many of these male UNP livers were noticeably more fatty in appearance on tissue collection, although the fat content of livers has not been empirically investigated in this study. Among females, the UNPs did not have the heaviest livers or the greatest Liver %BW – moreover, there was no significant difference between any of the female groups.

3.4.3 Puberty

All states of maternal undernutrition, whether prenatal and/or lactational, advanced the age of pubertal onset compared with Controls, in both females and males. The addition of a post-weaning HF diet further accelerated puberty – within all female groups, and in CONT and UNL groups in males. These data are consistent with clinical observations reported from many countries, that earlier menarche is associated with lower birth weight and accelerated growth in childhood (see Introduction). Given that maternal undernutrition during early critical windows of development leads to significant changes in offspring body composition and metabolic profile, it is perhaps not surprising that reproductive functioning might also be affected. A link between early nutrition and age of reproductive maturation has been postulated from studies of international adoption: girls adopted from underprivileged backgrounds but raised in developed countries experienced earlier onset of puberty compared to matched girls in their countries of origin (330, 331). Moreover, leptin has a role in the onset of puberty (281, 282): leptin restored puberty and fertility to *ob/ob* mice, which lack the gene for leptin synthesis and are normally infertile (332), and exogenous leptin administered after post-weaning advanced puberty in normal rodents (333, 334). Cunningham *et al* (1999) wrote: "Energy availability influences reproductive fitness. The activity of the reproductive axis is sensitive to the adequacy of nutrition and the stores of metabolic reserves … Leptin is a metabolic signal to the neuroendocrine reproductive system" p.216, (281). This is in line with the thrifty phenotype hypothesis and with the PAR understanding of development. Malnutrition during critical windows of development shapes metabolic functioning and energy use, and the changes produced by this phenotypic thrift are predictive and adaptive toward an anticipated postnatal environment. Undernutrition in early development signals and shapes the necessity for efficient storage of surplus energy in fat depots, and it may equally cue early pubertal maturation so as to maximise reproductive opportunities in an environment of anticipated deprivation.

Leptin may have a linked role in this cuing of both metabolic and reproductive axes. Rodents experience a neonatal leptin surge, for example, peaking around age P10 during lactation, and essential for development of the hypothalamic appetite centres (277, 335). Maternal undernutrition has been shown to drastically reduce this leptin surge along with the outgrowth of hypothalamic neuronal projections (336). Moreover, injections of leptin to female offspring of undernourished rat dams during days P3-P13 of lactation normalised body weight, adiposity and insulin and leptin levels in adulthood (123). Leptin helps regulate both energy balance and reproductive maturation, and may act as a coordinator, signalling between adequacy of energy reserves and the potential for reproductive success.

3.4.4 Conclusions

This study demonstrates that specific critical windows of undernutrition resulted in differential gender specific alterations in adiposity and adipoinsular axis function. Overall, the adverse metabolic effects displayed by offspring of dams fed 50% undernutrition during gestation were ameliorated in those offspring that continued to be undernourished into the period of lactation, that is, the UNPL cohort. Work by Desai *et al.* (2007) showed that delayed catch-up growth in IUGR offspring was beneficial in the prevention of adult obesity, but was complicated by significant adverse effects on pancreatic function (337). In the present study there were no significant changes in fasting insulin concentrations across Chow-fed offspring, and no changes in glucose levels. This difference could relate to the timing of the maternal nutritional challenge with the Desai study starting the paradigm of undernutrition at mid-gestation.

The present study utilised a balanced experimental protocol that permitted identification of the window of exposure-dependent outcomes resulting from moderate undernutrition to pregnant and lactating rats. The data clearly show that the level of nutrition available in pregnancy and lactation plays a major role in determining offspring metabolic phenotype. The greatest effects and those most likely to be harmful to long-term function (e.g. obesity and the metabolic syndrome) occur when pups whose mothers were restricted during pregnancy received a normal – and hence abundant compared with restricted – diet during the period of lactation. Following gestational undernutrition, maintenance of the level of undernutrition into the period of lactation conferred protective effects, particularly in females, on metabolic sequelae, and further highlights the possible adverse consequences associated with catch-up growth.

Moreover, maternal nutritional deprivation also programmed reproductive maturation in offspring. This was independent of the window of maternal undernutrition, since all periods of undernutrition, whether during pregnancy and/or lactation, resulted in advancement of pubertal onset. It thus appears that the programming effect of the early nutritional environment extends beyond offspring growth trajectory, fat deposition and metabolic phenotype. It is a broader phenomenon that may influence other important physiological regulators, such as those that promote pubertal onset and earlier readiness for reproductive opportunity.

Chapter 4: Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet

4.1 Background

The previous chapter utilised a model of moderate maternal undernutrition to examine critical windows of nutrient restriction and postnatal outcomes. Initial epidemiological studies suggested that fetal growth restriction is correlated with later disease, implying that fetal nutritional deprivation may be a strong programming stimulus. This prompted the development of experimental animal models using controlled maternal calorie, protein or macronutrient deficiency during key periods of development. However, in many societies, maternal and postnatal nutrition are either sufficient or excessive. As a result, excessive weight gain and/or obesity are one of the more common nutritional problems complicating pregnancy in developed countries.

In view of the rising prevalence of obesity in pregnancy and the association with gestational diabetes, there is now increasing interest in the detrimental influence of maternal obesity and excess maternal nutrition on the risk of disease in childhood and beyond (338-341). However, to date, relatively few studies have investigated long-term consequences of maternal nutrient excess during pregnancy or lactation on development of obesity in the offspring (131, 342, 343).

Within human populations, problems with maternal obesity are well documented – the incidence of gestational diabetes rises, as does pre-eclampsia and rates of caesarean section. Moreover, these effects may be self-perpetuating, as offspring of overweight mothers are themselves prone to obesity in adulthood thus giving rise to transgenerational effects. Although maternal obesity in the human is often paralleled by an increased risk for fetal macrosomia, there is also a growing body of evidence suggesting that obese mothers are characterised by an increasing prevalence of IUGR infants compared to non-obese mothers. The present study was therefore designed to investigate the effects of a moderate maternal HF diet during the pre-conceptional period and/or throughout pregnancy and lactation on birth phenotype and risk of obesity and related metabolic sequelae in male and female offspring in adult life when fed either a Chow or postnatal HF diet.

4.2 Materials and Methods

The animal model and endpoint measurements were detailed in Chapter 2 and are re-iterated briefly here.

4.2.1 Animal model

Male and female Wistar rats were acquired at a weaning age (22 days) and housed two per cage under standard conditions with a 12:12 light dark cycle and free access to water. Females were weight-matched and assigned to receive either standard rat Chow (*n*=16, Diet 2018, Harlan Teklad, Oxon) or a HF diet (*n*=8, 45% kcals as fat, D12451, Research Diets, NJ, USA) to be fed *ad libitum* for the duration of the trial. Males were fed Chow *ad libitum* for the duration of the pre-mating period. Body weights were recorded every 3 days from weaning until postnatal day 120. Acquiring of animals as weaners allowed a long period of familiarisation with handling and the research team prior to implementing the mating protocol, and thus minimised extraneous stressors.

At postnatal day 110, body composition in females was quantified using dual energy x-ray absorptiometry (DEXA, Lunar Prodigy, GE Medical Systems, Madison, WI, USA) as described previously. At postnatal day 120, females were time-mated using an estrus cycle monitor (Fine Science Tools, USA). Qualitative measures of the effect of the HF diet on estrus cycling were made by observation. Upon confirmation of mating, three maternal dietary groups were established: (1) Controls (CONT): females fed a standard Chow diet throughout their life and maintained on a standard Chow diet throughout pregnancy and lactation; (2) maternal high fat diet (MHF): females fed a HF diet throughout their life and maintained on the HF diet throughout pregnancy and lactation; and (3) pregnancy + lactation HF diet (PLHF): females fed a standard Chow diet until conception and then a HF diet throughout pregnancy and lactation. All pregnant dams were weighed and had food intakes measured daily throughout pregnancy. Following birth, pups were weighed, had lengths recorded and litter size was randomly adjusted to 8 pups (4 males and 4 females) to ensure standardized nutrition until weaning. Non-assigned pups were killed by decapitation at postnatal Day 2 (P2) and plasma samples pooled for later analysis. Lactating dams had body weights and food intakes measured throughout the lactation period and pups were weighed every three days until weaning.

After weaning, dams were fasted overnight and killed by decapitation following anaesthesia with sodium pentobarbitone (60mg/kg, IP) and plasma samples collected for insulin and leptin analyses. At weaning (P22), male and female offspring were housed 2 per cage (2 per

litter/gender/maternal background) and randomly assigned to receive either the standard Chow or the HF diet *ad libitum* until the end of the trial. From postnatal day 27, puberty was checked daily in male and female offspring using balanopreputial separation and vaginal opening. At postnatal day 150, animals (*n*=10-12 per group) had body composition quantified by DEXA scanning while under light isoflurane (2%) anaesthesia. At postnatal day 175 (P175), animals were fasted overnight and killed by decapitation following anaesthesia with sodium pentobarbitone (60mg/kg, IP). Blood was collected into heparinised vacutainers, centrifuged and plasma supernatant stored for future analysis. All animal experiments were approved under guidelines of the Animal Ethics Committee at the University of Auckland (R402).

4.2.2 Plasma analyses

Leptin and insulin concentrations were analysed on plasma from male and female pups at P2, lactating dams at postnatal day 22 (weaning), and postnatal male and female offspring at P160 (time of cull), using commercial rat-specific ELISAs (CrystalChem 90040 and 90060 respectively, CrystalChem Inc., Downers Grove, IL, USA). Fasting plasma glucose concentrations were measured using a glucose meter at the time of cull (Roche AccuChek).

4.2.3 Statistical analysis

Data for pups (day P2) were analysed by two-way factorial ANOVA with maternal background and gender as factors. Data for postnatal animals (P175) were analysed by three-way factorial ANOVA with maternal background, postnatal diet and gender as factors. Data on lactating dams were analysed using one-way ANOVA with maternal dietary background as a factor. Data from adult age-matched control female offspring (non-lactating females) were used to investigate effects of lactation. Analysis was performed using StatView statistical software (SAS, USA). All data are presented as means ± SEM unless otherwise stated.

4.3 Results

4.3.1 Maternal body weights

Dams raised on a HF diet from weaning (MHF) had significantly increased body weights (CONT 265 ± 3.5g *versus* MHF 295 ± 1.2g, $P<0.05$), and percentage total body fat compared to females raised on Chow (CONT and PLHF) at the time of mating (Figure 4.1).

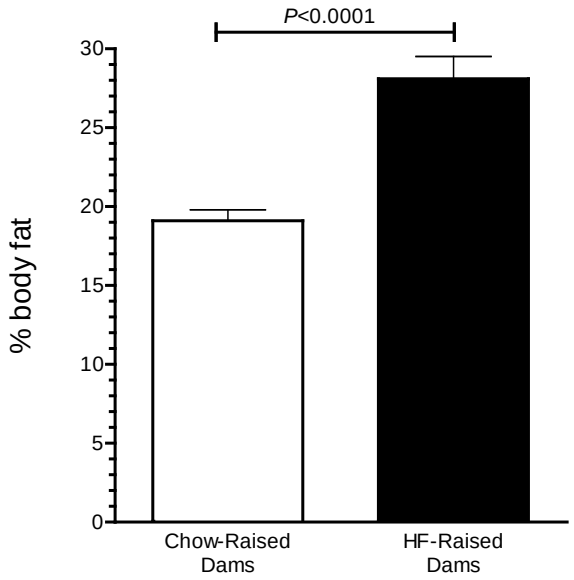


Figure 4.1: Percentage of total body fat of dams at pre-conception, as quantified by DEXA scanning of chow-raised dams (n = 40) and dams raised on a high fat (HF) diet (n = 20). Chow-raised dams included control dams and dams that would go on to be fed the HF diet during pregnancy and lactation. Data are means ± SEM.

Of note, the HF diet resulted in reduced cycling (as monitored over 3 cycle periods i.e. 12 days via the daily estrus probe method) in MHF dams compared to Chow-fed animals (CONT 95% cycling, MHF 45% cycling). Only 9 out of 20 MHF dams were successfully mated, in contrast to 38 out of 44 successful matings among the Chow-raised females.

4.3.2 Caloric intake during pregnancy

Energy intake during gestation (kcals/g body weight) was not different between CONT and MHF dams. Caloric intake was significantly increased in the PLHF dams during the first week of gestation compared to CONT and MHF dams, but normalised to that of CONT dams from Day 9. (Figure 4.2 and Table 4.1).

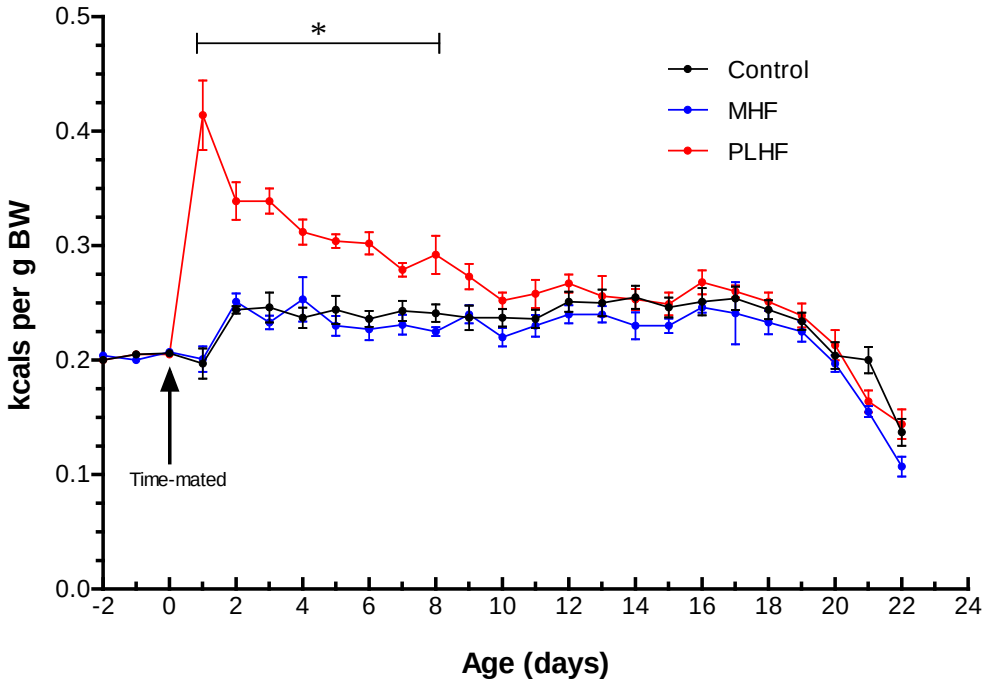


Figure 4.2: Energy intake (kcal/g body weight) during pregnancy in chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Data are means ± SEM. * *P*<0.0001 PLHF *vs* CONT and MHF (Two Way Repeated Measures ANOVA with maternal diet and age as factors).

Table 4.1: Energy Intake (total kcal per 7-day period) consumed per dam during gestation

Group (dams)	Gestation, Days 1-7	Gestation, Days 8-14	Gestation, Days 15-21
CONT	481 ± 11	535 ± 12	596 ± 17
MHF	524 ± 10	503 ± 14	605 ± 16
PLHF	659 ± 15	592 ± 12	609 ± 15
CONT *vs* MHF	NS	NS	NS
CONT *vs* PLHF	*P*<0.0001	*P*<0.05	NS
MHF *vs* PLHF	*P*<0.0001	*P*<0.05	NS

Data are means ± SEM; *n* = 8-12 dams per group.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.

4.3.3 Maternal weight gain

There were no significant differences in maternal weight gain across the treatment groups during gestation, although the MHF dams remained significantly heavier than the other groups. (Figure 4.3).

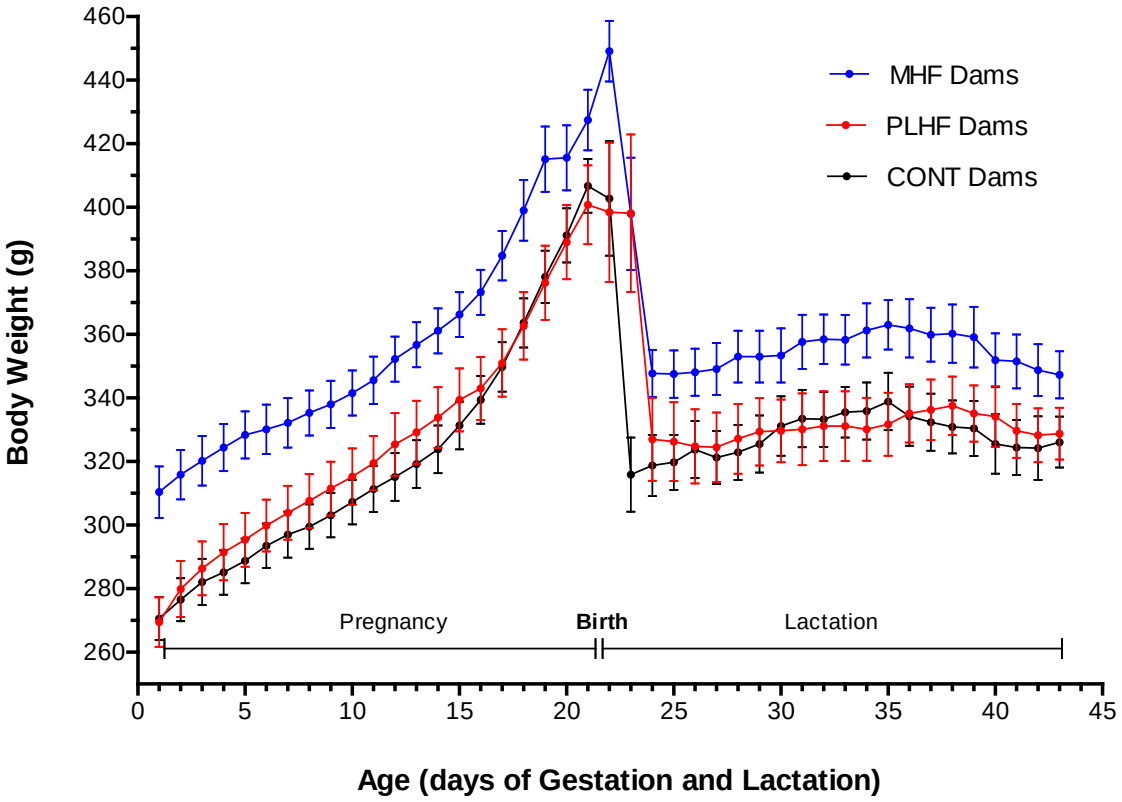


Figure 4.3: Body weights during pregnancy and lactation of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Data are means ± SEM. *P*<0.001 MHF *vs* CONT and PLHF (One Way ANOVA with maternal diet as factor).

The mean absolute total weight gain across Days 1-21 of pregnancy, for each group of dams was:

CONT	136 ± 5.3g
MHF	132 ± 3.4 g
PLHF	142 ± 6.1 g

CONT *vs* MHF *vs* PLHF = NS

(Data are means ± SEM. One Way ANOVA with maternal diet as factor.)

4.3.4 Length of gestation

Length of gestation was increased in the two maternal HF dietary groups by approximately 24 hours – particularly noticeable in the PLHF dams (Table 4.2).

Table 4.2: **Length of gestation**

Treatment Group (dams)	Gestation (days)
CONT	21.6 ± 0.14
MHF	22.5 ± 0.18
PLHF	22.8 ± 0.14
CONT *versus* MHF	*P*<0.05
CONT *versus* PLHF	*P*<0.05
MHF *versus* PLHF	NS

Data are means ± SEM; *n* = 8-12 dams per group.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.

4.3.5 Birth weights

A maternal HF diet led to a small but significant reduction in birth weight in male and female pups compared to Controls. The PLHF pups were the lightest group, and this difference was significant against both CONT and MHF groups. (Table 4.3). There was no difference in litter size or sex ratio compared to Controls (data not shown).

Table 4.3: **Birth weights**

Group (dams)	Birth Weights, Males (g)	Birth Weights, Females (g) *
CONT	6.3 ± 0.07	6.1 ± 0.06
MHF	5.9 ± 0.05	5.7 ± 0.05
PLHF	5.5 ± 0.04	5.3 ± 0.03
CONT *vs* MHF	*P*<0.0005	*P*<0.0005
CONT *vs* PLHF	*P*<0.0001	*P*<0.0001
MHF *vs* PLHF	*P*<0.05	*P*<0.005

Data are means ± SEM.
n = 29-37 neonates per group, after runts were excluded and litter sizes standardised.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.
*Females were overall lighter than males, *P*<0.001 (Two Way ANOVA with maternal diet and gender as factors.)

4.3.6 Growth of pups from birth to weaning

During lactation MHF and PLHF offspring gained more weight than CONT offspring. MHF and PLHF growth curves overtook that of the CONT group, and then continued to diverge from it.

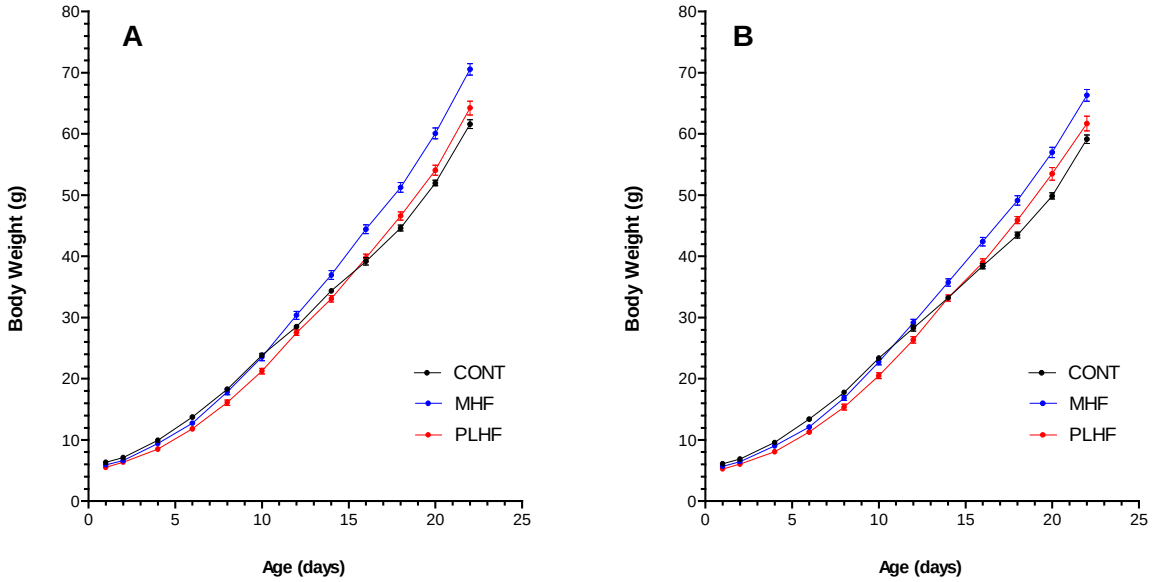


Figure 4.4: Weight gain during lactation in male (A) and female (B) offspring of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Data are means ± SEM. *P*<0.05 MHF *vs* CONT and PLHF (One Way Repeated Measures ANOVA, with maternal diet as factor).

Final weaning weights were slightly but significantly increased in MHF and PLHF male and female offspring compared to controls indicative of catch-up growth (Table 4.4).

Table 4.4: **Weaning Weights (P22)**

Group (dams)	Weaning Weights, Males (g)	Weaning Weights, Females (g) *
CONT	61.6 ± 0.7	59.1 ± 0.7
MHF	70.5 ± 0.9	66.3 ± 0.9
PLHF	64.2 ± 1.2	63.8 ± 0.8
CONT *vs* MHF	*P*<0.0001	*P*<0.0001
CONT *vs* PLHF	*P*<0.05	*P*<0.05
MHF *vs* PLHF	*P*<0.05	*P*<0.05

Data are means ± SEM; *n* = 26-37 pups per group.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.
*Females were overall lighter than males, *P*<0.001 (Two Way ANOVA with maternal diet and gender as factors.)

4.3.7 Caloric intake during lactation

All three dietary treatment groups increased their energy intake during lactation (Figure 4.5). MHF and PLHF dams exhibited an identical increasing linear intake. CONT dams also maintained this same energy intake until Day 34, but then their curve separated for 6 days (possibly reflective of increased energy intake demand from the MHF and PLHF offspring) before returning to the same level as the MHF and PLHF dams by weaning.

N.B: data from the last 2 days pre-weaning are excluded due to confounding of food intake data by the additional intake of weaners.

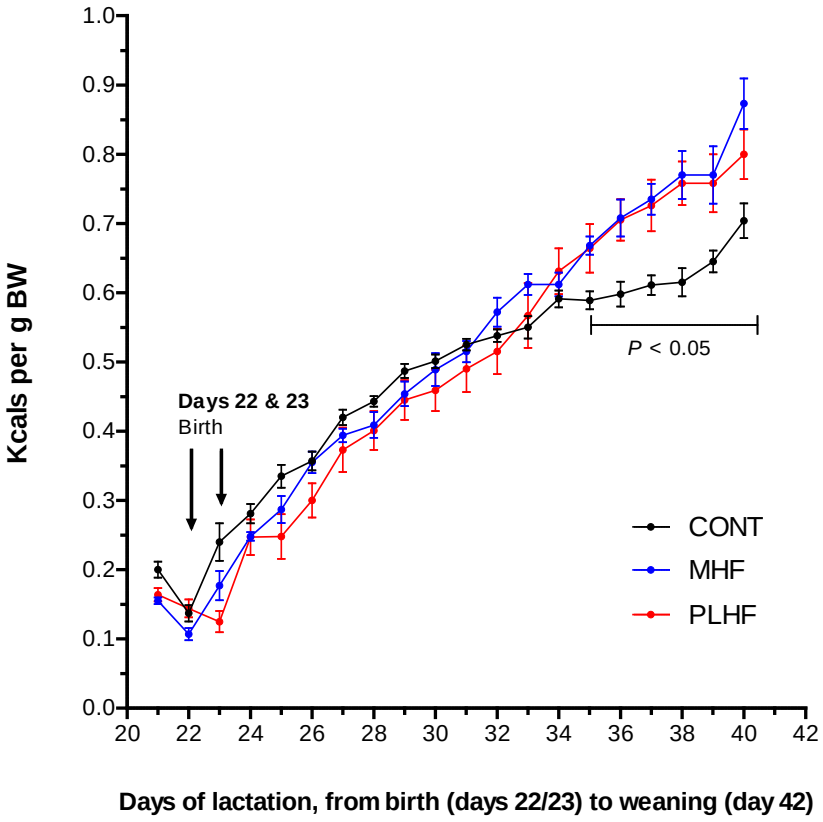


Figure 4.5: Energy intake (kcal/g body weight) during lactation of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Data are means ± SEM. *P*<0.05 CONT *vs* MHF and PLHF day 35 – 41 (Two Way Repeated Measures ANOVA with maternal diet and age as factors).

4.3.8 Post-weaning weight gain

A postnatal HF diet significantly increased body weight gain in all groups. Postnatal body growth was significantly increased in male and female offspring of MHF and PLHF dams, independent of postnatal diet (Figure 4.6, A-D). By postnatal day 150, male and female offspring of MHF and PLHF-fed dams were significantly heavier than CONT animals on both Chow and HF diets.

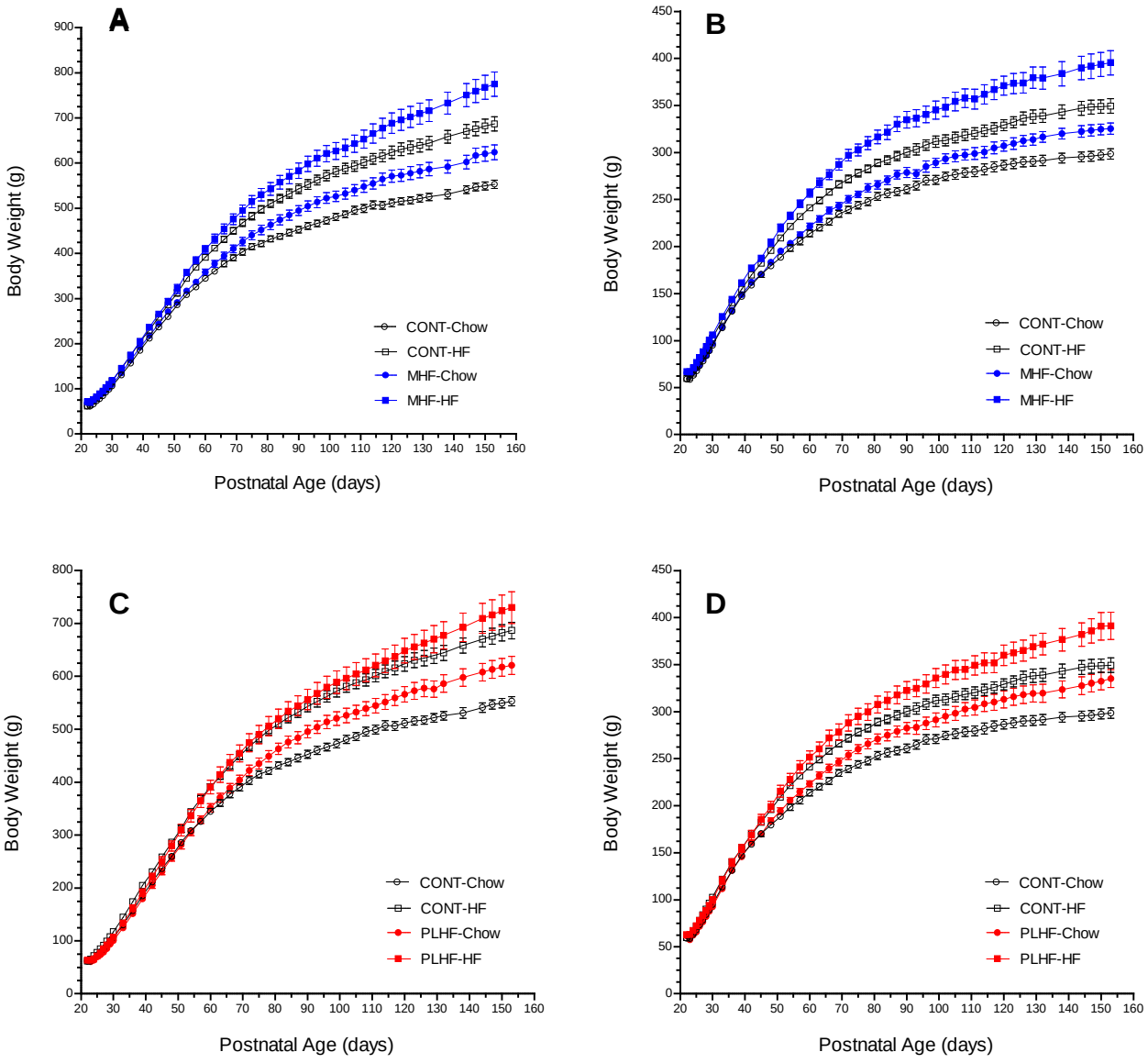


Figure 4.6: Post-weaning growth curves. (A) Male and (B) female offspring of chow-fed control dams (CONT) and dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), raised on chow or a high fat (HF) diet after weaning. (C) Male and (D) female offspring of CONT dams and dams fed a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF), raised on chow or a HF diet after weaning. Data are means ± SEM, *n* = 12-18 per group. *P*<0.001 for maternal diet effect and postnatal diet effect (Three Way ANOVA with maternal diet, postnatal diet and gender as factors). There were no effect interactions.

4.3.9 Post-weaning energy intake in offspring

Total energy intake (kcals/g body weight) was measured at postnatal days 23-30, 42, 64, 96 and 153, in both male and female offspring (representing pre-pubertal, early post-pubertal, adult and mature adult time points). Energy intake rose sharply at weaning in all animals, peaked within 4-6 days, then gradually declined with age. Irrespective of postnatal diet, Chow *versus* HF, there was no overall significant difference in energy intake curves between any groups, either within males or females. Significant differences did emerge within some same-day comparisons, but these were present only very early on in post-weaning life (within the first 1-4 days), after which there were no significant differences in caloric intake across the treatment groups, within genders, at the ages measured. (Two Way Repeated Measures ANOVA, with factors Maternal Diet and Age (within Genders), and factors Postnatal Diet and Age (within Groups). Data not shown.)

4.3.10 Onset of puberty

The offspring of the two maternal HF dietary treatment groups (MHF and PLHF) both exhibited significant earlier onset of puberty, in males and in females ($P<0.001$). A post-weaning HF diet further advanced onset of puberty in both CONT males and females ($P<0.001$), but had no additive effect in either gender of the MHF or PLHF groups. (See Table 4.5 and Figure 4.7)

Table 4.5: **Age and weight at puberty**

	Males		**Females**	
	Pubertal Age (days)	Pubertal Wgt (g)	Pubertal Age (days)	Pubertal Wgt (g)
CONT-Chow	37.4 ± 0.4	170.3 ± 4.1	34.6 ± 0.5	121.5 ± 3.7
CONT-HF	35.4 ± 0.6	173.6 ± 6.7	32.8 ± 0.4	116.7 ± 6.3
MHF-Chow	34.1 ± 0.3	143.4 ± 3.4	33.0 ± 0.3	114.4 ± 2.4
MHF-HF	34.0 ± 0.4	155.6 ± 5.4	32.6 ± 0.7	122.5 ± 5.0
PLHF-Chow	35.0 ± 0.5	142.1 ± 4.9	32.7 ± 0.4	108.6 ± 2.7
PLHF-HF	34.2 ± 0.6	143.5 ± 5.2	31.4 ± 0.5	103.8 ± 3.8

Data are means ± SEM.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.
P=0.001 for maternal diet effect in both males and females
P=0.001 for postnatal diet effect in both males and females

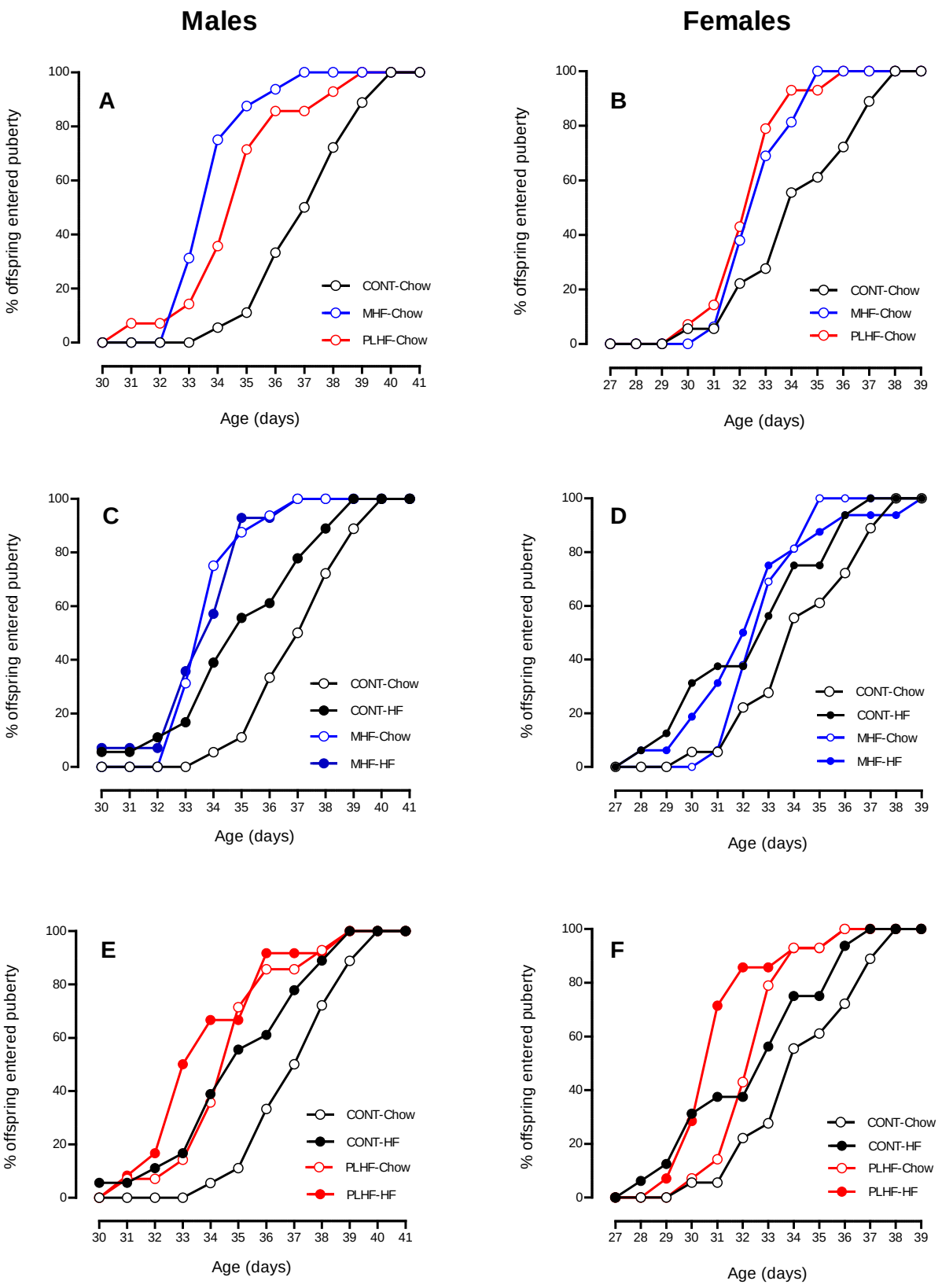


Figure 4.7: Age of pubertal onset (% of offspring entering puberty over time). (A) Male and (B) female chow-fed offspring of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), and dams fed a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). *P*<0.001 for maternal diet effect in both males and females. (C) Male and (D) female offspring of CONT and MHF dams, fed either chow or a HF diet after weaning. (E) Male and (F) female offspring of CONT and PLHF dams, fed either chow or a HF diet after weaning. *P*<0.001 for postnatal diet effect in both male and female offspring of the CONT dams. Data are percentage of total pups entered puberty, *n* = 12-18 pups per group. (Three Way ANOVA with maternal diet, postnatal diet and gender as factors.)

Body Weight at Puberty MHF and PLHF offspring all showed significantly lower body weights at pubertal onset compared to CONT offspring, within Chow-fed and HF-fed groups, in both males and females (Table 4.5)
(Three Way ANOVA, with Maternal Diet, Postnatal Diet and Sex as factors.)

4.3.11 Body composition

Adiposity (percentage total body fat mass, as determined by DEXA) was significantly increased in the offspring of the maternal HF treatment groups, both males and females, even when fed a Chow diet postnatally (Figure 4.8, A&B). Percentage body fat was further increased in all animals fed a HF diet postnatally. There were no significant differences in body fat between MHF and PLHF offspring. There was a highly significant Maternal Diet x Postnatal Diet interaction in males (P=0.006), but not in females (P=0.870). Female offspring fed the HF diet displayed the same relative increase in adiposity over their Chow-fed siblings, independent of maternal dietary group (Figure 4.8, C). However, CONT males showed a marked increase in adiposity when raised on HF compared to the MHF and PLHF offspring, where the response to HF feeding was reduced relative to that of Chow-fed animals (Figure 4.8, C).

Note: due to logistical considerations and the size of the experimental cohort, not all animals were DEXA scanned. However, a minimum of 8 animals per group were scanned and DEXA data tightly correlated with retroperitoneal fat pad data, thus confirming the reliability of the data from the DEXA-scanned subcohort.

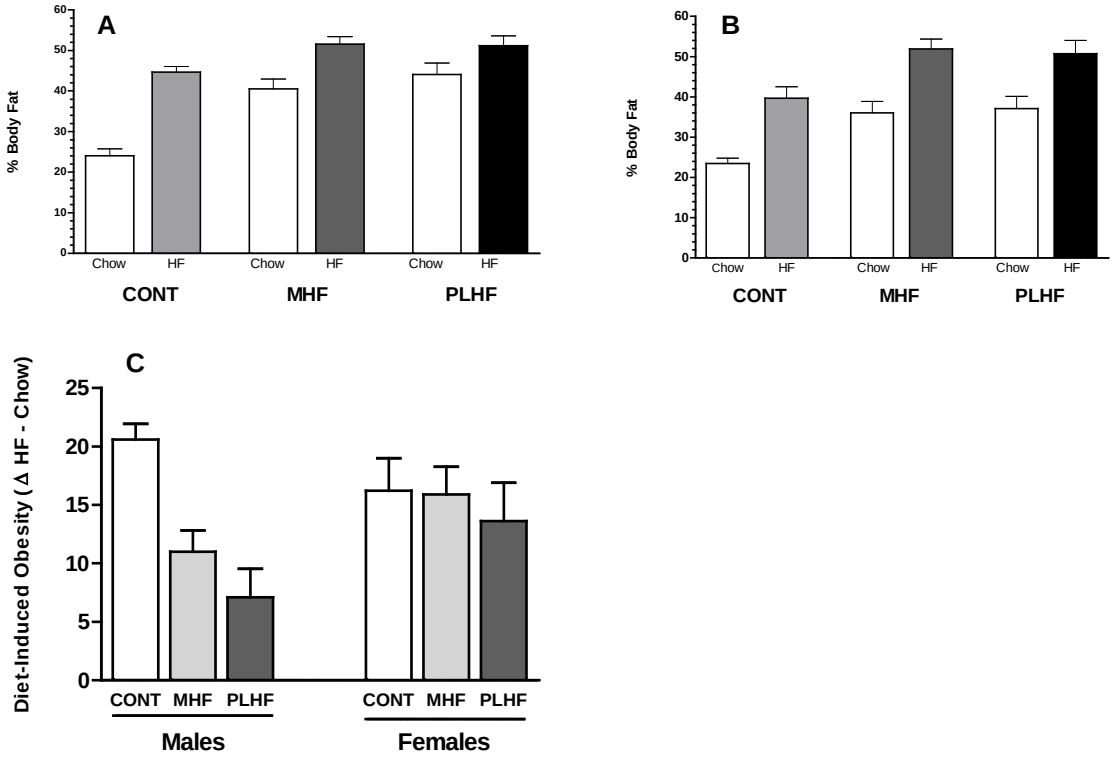


Figure 4.8: Adiposity (percent total body fat as quantified by DEXA scanning) and diet-induced obesity (DIO) in male and female offspring of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), and dams fed a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF) at postnatal day 150. Adiposity in (A) male offspring and (B) female offspring. $P<0.001$ for both maternal diet effect and postnatal diet effect. There were no effect interactions (Two Way ANOVA with maternal diet and postnatal diet as factors). (C) Diet-induced obesity (% body fat: difference between means of HF-fed animals and Chow-fed animals for each maternal dietary group). $P<0.05$ CONT *vs* MHF and PLHF in males only. $P<0.05$ for male-female interaction (Two Way ANOVA with maternal diet and gender as factors). Data are means ± SEM, n = 8-10 per group.

4.3.12 Plasma insulin and leptin concentrations

Maternal: Fasting leptin concentrations in lactating dams at P22 (at the time of weaning) were significantly lower when compared to control age-matched HF-fed non-lactating females (NLF). Of note, plasma leptin levels in lactating dams were not affected by a HF diet. Maternal fasting plasma insulin concentrations however were significantly increased in MHF and PLHF dams compared to CONT dams ($p<0.05$), and were further increased in PLHF dams compared to MHF dams. Fasting plasma insulin concentrations were higher in lactating PLHF dams compared to age-matched NLF-Chow and NLF-HF animals, but were not different between the other groups. (Table 4.6).

Table 4.6: **Plasma leptin and insulin in lactating dams and neonates (P2)**

Group	Offspring, P2 (ng/ml)		Dams, Day 22, Lactation	
	Leptin	Insulin	Leptin	Insulin
CONT	5.50 ± 1.60 [a]	2.9 ± 0.62 [a]	1.71 ± 0.19 [a]	1.91 ± 0.4 [a,d]
MHF	1.54 ± 0.49 [b]	1.9 ± 0.25 [b]	1.86 ± 0.38 [a]	3.18 ± 0.6 [b]
PLHF	1.58 ± 0.33 [b]	1.6 ± 0.23 [b]	2.55 ± 0.77 [a]	4.53 ± 0.7 [c]
NLF-Chow	- -	- -	3.47 ± 0.31 [a]	1.14 ± 0.1 [a]
NLF-HF	- -	- -	7.52 ± 1.07 [b]	2.42 ± 0.2 [d]

Data are means ± SEM; *n* = 8-12 per group (dams), 6-8 neonates per litter.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.
Means with different letters are significantly different from each other (*P*<0.05).
Neonatal plasma was pooled from male and females within litter.

Plasma leptin in offspring: Plasma leptin concentrations in pups at P2 were significantly reduced in offspring of MHF and PLHF dams compared to CONT dams (*P*<0.001, Table 4.6). There were no significant differences in fasting leptin concentrations between MHF and PLHF offspring at P2.

In contrast, in adulthood, fasting plasma leptin concentrations were significantly increased in MHF and PLHF male and female Chow-fed offspring compared to CONT offspring and were increased even further if offspring were fed a postnatal HF diet (Figures 4.9, A&B). Fasting leptin concentrations positively correlated with fat mass across all treatment groups ($r^2 = 0.72$, *P*<0.0001).

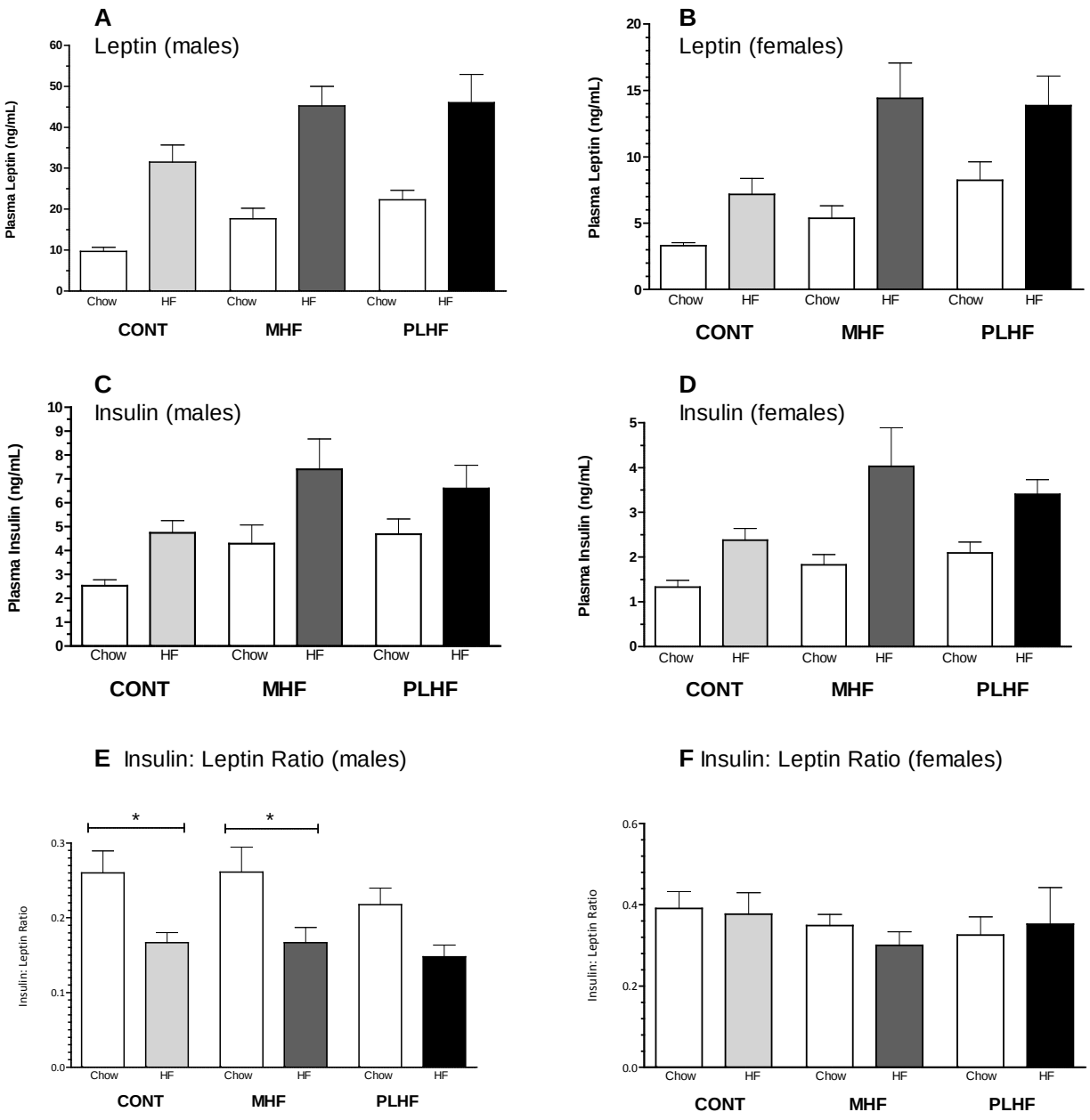


Figure 4. 9: Fasting leptin (A and B) and insulin concentrations (C and D) and insulin:leptin ratios (E and F) in chow or high fat (HF)-fed offspring of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), and dams fed a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF) at postnatal day 160. (A and B): *P*<0.001 for maternal diet effect and postnatal diet effect in both males and females. (C and D): *P*<0.05 for maternal diet effect; *P*<0.001 for postnatal diet effect in both males and females. (E and F): *P*<0.001 for postnatal diet effect in males only (*). (Two Way ANOVA with maternal diet and postnatal diet as factors). Data are means ± SEM, *n* = 8-12 per group.

Offspring Insulin and Glucose As with leptin, plasma insulin concentrations in pups at P2 were significantly reduced in MHF and PLHF offspring compared to CONT offspring, and were not different between MHF and PLHF offspring (P<0.001, Table 4.6).

At P160, fasting plasma insulin concentrations were significantly higher in MHF and PLHF male and female Chow-fed offspring compared to CONT offspring, and were increased even further following a postnatal HF diet (Figures 4.9, C&D). There were no significant differences in fasting insulin concentrations between MHF and PLHF offspring.

Plasma glucose concentrations showed no effect of maternal diet, but were slightly but significantly elevated in MHF-HF over MHF-Chow males (see Table 4.7).

Table 4.7: **Plasma glucose concentrations (mmol/l)**

		Postnatal Diet		Significance
		Chow	HF	
Males	CONT	5.6 ± 0.2	6.0 ± 0.2	NS
	MHF	5.7 ± 0.3	6.4 ± 0.2	P = 0.016
	PLHF	5.7 ± 0.2	6.1 ± 0.1	NS
Females	CONT	5.6 ± 0.1	5.6 ± 0.1	NS
	MHF	5.4 ± 0.1	5.6 ± 0.1	NS
	PLHF	5.5 ±0.1	5.7 ± 0.2	NS

Data are means ± SEM; n = 8-12 per group.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat.

Offspring Insulin:Leptin Ratio There was no Maternal Diet Effect evident in the Insulin:Leptin Ratio, in either males or females. In males however, there was a Postnatal Diet Effect (P<0.001), significant within CONT and MHF groups; there was no Postnatal Diet Effect in the females (P=0.213). (Figure 4.9, E&F.)

4.3.13 Correlations

Correlations are a means of testing for a statistical relationship between variables. Where various assays are used to measure the same construct (for example, we had several assays for adiposity in our study), correlation tests may help determine which is the more accurate or sensitive assay.

The measures of adiposity correlated well within the Chow-fed offspring groups, but as total body fat increased (for example, because of postnatal diet-induced obesity) the retroperitoneal fat pad became a less sensitive indicator of adiposity. Correlations between the weight of the retroperitoneal fat mass and the DEXA data were uniformly significant only within the PLHF offspring, whereas the CONT and MHF HF-fed groups distributed their adipose tissue differently, as evidenced by a postnatal diet disparity (Table 4.8, males only, female data similar).

In the Chow-raised groups, plasma leptin consistently correlated with all measures of adiposity. In the PLHF animals, this strong correlation continued into the postnatal HF-fed group. However, this postnatal diet continuity was not evident in the HF-fed CONT or MHF groups, where levels of circulating leptin did not match degree of adiposity.

Table 4.8: **Correlations between adiposity, plasma leptin and plasma insulin of male offspring**

	CONT-Chow	CONT-HF	MHF-Chow	MHF-HF	PLHF-Chow	PLHF-HF
Total Body Fat (g) *vs* Wgt, retro fat pad (g) *	R^2 = 0.74 *P* = 0.001	R^2 = 0.27 *P* = 0.15	R^2 = 0.72 *P* = 0.007	R^2 = 0.06 *P* = 0.61	R^2 = 0.88 *P* = 0.0006	R^2 = 0.91 *P* = 0.0003
%Body Fat *vs* Wgt of retro fat pad as % total body wgt *	R^2 = 0.68 *P* = 0.003	R^2 = 0.02 *P* = 0.74	R^2 = 0.36 *P* = 0.12	R^2 = 0.12 *P* = 0.45	R^2 = 0.73 *P* = 0.007	R^2 = 0.80 *P* = 0.003
plasma Leptin *vs* %Body Fat	R^2 = 0.81 *P* = 0.006	R^2 = 0.14 *P* = 0.40	R^2 = 0.91 *P* = 0.0009	R^2 = 0.26 *P* = 0.25	R^2 = 0.72 *P* = 0.008	R^2 = 0.71 *P* = 0.009
plasma Leptin *vs* Total body fat (g)	R^2 = 0.77 *P* = 0.009	R^2 = 0.04 *P* = 0.62	R^2 = 0.98 *P* < 0.0001	R^2 = 0.43 *P* = 0.11	R^2 = 0.76 *P* = 0.005	R^2 = 0.86 *P* = 0.0008
plasma Leptin *vs* Wgt, retro fat pad (g)	R^2 = 0.37 *P* = 0.008	R^2 = 0.28 *P* = 0.02	R^2 = 0.55 *P* = 0.003	R^2 = 0.29 *P* = 0.055	R^2 = 0.56 *P* = 0.002	R^2 = 0.77 *P* = 0.0002
plasma Insulin *vs* %Body Fat	R^2 = 0.29 *P* = 0.22	R^2 = 0.003 *P* = 0.90	R^2 = 0.26 *P* = 0.24	R^2 = 0.03 *P* = 0.73	R^2 = 0.57 *P* = 0.03	R^2 = 0.17 *P* = 0.31
plasma Insulin *vs* Total body fat (g)	R^2 = 0.19 *P* = 0.33	R^2 = 0.0007 *P* = 0.95	R^2 = 0.09 *P* = 0.51	R^2 = 0.43 *P* = 0.11	R^2 = 0.37 *P* = 0.11	R^2 = 0.30 *P* = 0.16
plasma Insulin *vs* Wgt, retro fat pad (g)	R^2 = 0.0006 *P* = 0.94	R^2 = 0.06 *P* = 0.39	R^2 = 0.15 *P* = 0.24	R^2 = 0.002 *P* = 0.89	R^2 = 0.01 *P* = 0.68	R^2 = 0.04 *P* = 0.52
plasma Leptin *vs* plasma Insulin	R^2 = 0.17 *P* = 0.16	R^2 = 0.52 *P* = 0.002	R^2 = 0.44 *P* = 0.03	R^2 = 0.44 *P* = 0.03	R^2 = 0.23 *P* = 0.08	R^2 = 0.41 *P* = 0.03

Significant correlations are shaded grey.
CONT = control; MHF = maternal high fat; PLHF = pregnancy and lactation high fat; chow = chow-fed; HF = high fat-fed.
* Correlations between measures of adiposity were only calculated between those with same units of measurement: that is, comparing grams *versus* grams, % *versus* %.

There was no relationship between adiposity and plasma insulin.

All six groups of male offspring exhibited a relationship between plasma leptin and plasma insulin, with all groups either reaching or tending toward statistical significance (see Table 5.8). Of note, the data shows that plasma leptin and insulin were more strongly related in the HF-fed animals. Moreover, the relationship between the means of plasma leptin and insulin was similar across groups; that is, although the MHF and PLHF offspring exhibited hyperinsulinemia and hyperleptinemia compared to Controls, the two circulating hormones remained in linear relationship with each other, see Figure 4.10 (Chow-fed males only; female data similar.) This relationship is also evident in the Insulin:Leptin Ratio depicted in Figure 4.9E&F, where the ratio between the two hormones remains the same between groups fed the same postnatal diet.

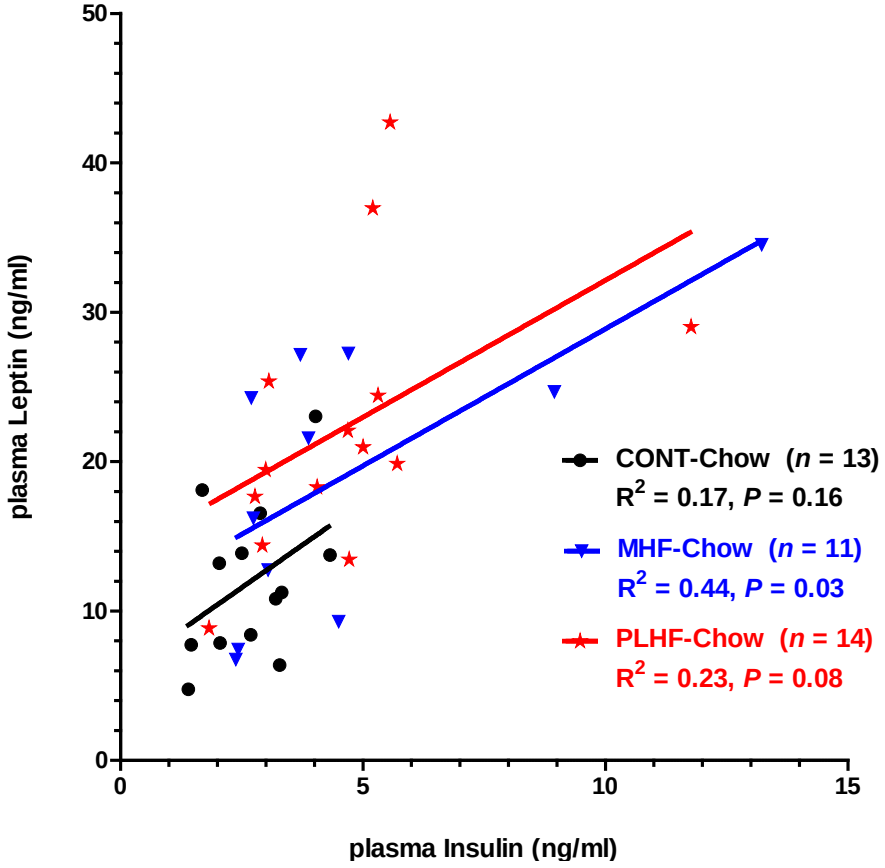


Figure 4.10: Correlations between plasma leptin and plasma insulin in chow-fed male offspring of chow-fed control dams (CONT), dams fed a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), and dams fed a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). *n* = 11-14.

In female offspring, plasma insulin and plasma leptin correlated in MHF and PLHF Chow-fed animals, but not in CONT. There was no correlation in any of the female HF-fed offspring. (Females, plasma insulin *vs* plasma leptin, CONT-Chow: $R^2 = 0.30$, $P = 0.20$; MHF-Chow: $R^2 = 0.35$, $P = 0.02$; PLHF-Chow: $R^2 = 0.42$, $P = 0.01$; CONT-HF: $R^2 = 0.10$, $P = 0.24$; MHF-HF: $R^2 = 0.18$, $P = 0.13$; PLHF-HF: $R^2 = 0.12$, $P = 0.23$; $n = 10$-18).

4.4 Discussion

In this part of the study we explored the effects of a maternal HF diet - applied during two contrasting windows of maternal life - on the development and long term health of the offspring. We compared these effects in male *versus* female offspring. We further investigated postnatal growth by challenging the offspring with two different post-weaning diets, either Chow or HF.

A distinct offspring phenotype emerged due to the programming effect of the maternal HF diet. Compared to Controls the two groups subjected to the maternal HF treatment (the MHF and PLHF offspring) were: small at birth, heavier at weaning, with increased weight gain irrespective of post-weaning diet. Increased body weight was due to increased adiposity. Moreover these rats, while normoglycemic, were hyperinsulinemic and hyperleptinemic in adulthood.

There tended to be few differences between the MHF and PLHF groups, which suggested that it was the HF diet during the period of pregnancy and lactation that exerted the predominant programming influence, whereas feeding HF preconception had less effect. Potential mechanistic differences in obese and hyperinsulinemic phenotype development at the level of the pancreas are discussed in detail in Chapter 5.

4.4.1 Comparing dams at mating: diet-induced obesity

The MHF dams, placed on a HF diet from the time they entered the study as weaners, exhibited considerable weight gain over the Chow-fed animals. By the age of mating (Day 100) they were significantly heavier. Such diet-induced obesity (DIO) is well documented (344-348) and in a range of species (349). It is known that rats defend a body weight set-point (350) and that long-term maintenance on HF diets will irreversibly adjust the body weight set-point upwards (351). These authors found the heavier weight was due to increased fat pad mass, containing a greater total number of adipocytes that were larger in size. Our DEXA scans confirmed that the heavier body weight of our HF-fed dams was due to increased fat as a percentage of total body mass.

At the time of mating an estrus probe was used to daily assess females to determine stage of estrus cycle. The HF diet resulted in reduced cycling (as monitored over three cycle periods) in MHF dams compared to Chow-fed animals (CONT 95% cycling, MHF 45% cycling).

4.4.2 Maternal energy intake during pregnancy and lactation

The three groups of dams – both Chow- and HF-fed - all had similar caloric intakes. Caloric intake was identical before mating, despite the different nutritional treatments under which the dams were raised. Then, as soon as pregnancy was confirmed, the PLHF cohort of dams underwent a dietary transition from Chow to HF. This transition produced a dramatic but temporary surge in PLHF energy intake during the early period of gestation, significantly increased compared to CONT and MHF dams. But then, by the second week of gestation, PLHF energy intake normalised back to the level of Controls, and remained thus through to parturition.

The energy intake of all three groups of dams was also identical for the first half of lactation. However, on the 11th-12th day into lactation, the caloric intake of the Control Chow-fed dams decreased compared to the two HF-fed groups which continued on the same steep trajectory.[2] This decreased level of energy intake by the CONT dams lasted 7 days before returning again to the same level as the MHF and PLHF dams. Other researchers have also reported similar calorific intake between HF- and Chow-fed dams (138, 352). These data suggest that rats possess mechanisms that tightly regulate caloric intake, irrespective of diet, and that consumption in rats is tuned to caloric intake.

There were no significant differences in maternal weight gain across the treatment groups during gestation (CONT 136 ± 5.3 g, MHF 132 ± 3.4 g, PLHF 142 ± 6.1 g) despite the MHF dams remaining significantly heavier than the other groups.

The paralleling of weight gain throughout pregnancy between the three cohorts of dams, and their identical caloric intakes strongly indicates that the dams on the HF treatment diet followed a normal adaptation to pregnancy. The spike in food consumption experienced by the PLHF group at the beginning of gestation represented a response to the transition in diet, not an abnormal adaptation to pregnancy. The rapid return to the common caloric intake of pregnancy – a little higher than the non-pregnant state – without disturbances to the typical weight gain of pregnancy, suggested that this HF treatment regime did not interfere with the normal pattern of physiologic adaptation to pregnancy. It also meant that the phenotype produced by maternal HF-feeding resulted from the exposure to dietary fat, and not from aberrations of caloric intake.

[2] Alternatively, it may be that rather than a decrease in CONT intake, there was increased intake by the HF dams and offspring, to meet the demands of catch-up growth occurring during this period.

Further evidence of a normal adaptation to pregnancy is found in the no difference seen in litter size or sex ratio *versus* Controls.

In developed countries, 15-20% of reproductive aged women are obese (353) and in a recent report, >40% of women gained more weight during pregnancy than is considered ideal (354, 355). Inappropriate pregnancy weight gain and maternal obesity have long-term effects on the developing offspring (356), who then go on to develop early puberty and have a greater risk for obesity – leading to a cycle of overweight mothers preparing their children for the same destiny. High gestational weight gain has been shown to significantly increase the risk of childhood obesity (340). Intriguingly, we demonstrate in the present study that the consumption of HF both prior to and including pregnancy did not increase maternal weight gain over and above control pregnancies. Although the MHF dams – fed a HF diet throughout their lifespan – entered pregnancy with heavier body weights and increased adiposity, their gestational weight gain profile was identical to the CONT dams. These data may suggest that it is the composition of the diet rather than maternal weight gain *per se* that has an effect on offspring phenotype.

4.4.3 Gestation length

We observed a significantly longer gestation length in dams fed a HF diet. These data are not dissimilar to those reported in human populations known to consume high levels of *n*-3 fatty acids (357-359), where *n*-3 fatty acids are thought to interfere with the production of prostaglandins necessary for the activation of parturition. The diet used in our study derives its fat from animal lard, which is primarily composed of oleic acid, an *n*-9 fatty acid. The impact of these fatty acids on gestation length is intriguing although the mechanisms are unclear and warrant further investigation.

At the end of lactation (P22) when dams were sacrificed, the MHF and PLHF dams were found to be hyperinsulinemic compared to CONT (with the PLHF insulin also elevated against MHF), but with no difference in plasma leptin levels between groups – despite differences in body weight and adiposity. In fact the MHF and PLHF dams were hypoleptinemic when compared with age matched non-lactating females (NLF) on similar HF diet. Lactation associated hypoleptinemia in the rat has been previously reported (360). These observations were associated with marked increases in food consumption during the late lactation period and may suggest that these endocrine changes are physiologically appropriate, to help meet the high metabolic demands of lactation (361). Hyperinsulinemia during lactation is associated with increased weight gain and risk of diabetes later in life (362).

4.4.4 Birth weight

In our experiment HF-feeding during pregnancy and lactation caused intrauterine growth retardation (IUGR) – pups born to the two maternal HF dietary treatment groups were slightly but significantly smaller than Controls. This finding is of interest – it is a common and contrary observation among humans that obese mothers give birth to larger babies, a condition known as macrosomia. Macrosomia is typically associated with gestational diabetes (GDM) (363, 364) and is caused by excess fetal insulin secreted as a response to maternal hyperglycemia (365, 366). In severely diabetic human mothers, however, microsomia exists – growth retarded neonates who are small for gestational age. This is thought to be the result of abnormally low fetal insulin levels due to fetal pancreatic islet cell exhaustion or abnormal formation secondary to maternal hyperglycemia. Hypoinsulinemia leads to a reduced number of fetal insulin receptors; glucose uptake and protein synthesis are impaired, resulting in IUGR and microsomia (150, 367).

In rodent models maternal HF feeding has been reported to have variable effects on birth weight, with some studies reporting no effects (138, 352, 368-371) while others reported either decreased (137, 372-375) or increased birth weights (135), although the last study used a high fat: high sugar diet.[3] These discrepancies are likely due to differing fatty acid composition of the fat enriched diets across studies with variable levels in maternal saturated fat intakes (135, 298). It has also been shown recently that maternal preconceptional obesity resulted in no differences in birth weights in offspring (371).

Although we did not measure maternal glucose and insulin levels throughout pregnancy in the present study, the fact that we demonstrated birth weight reduction rather than augmentation (macrosomia) would suggest that it is unlikely that the results we observed were due to the development of GDM. It is possible that the growth restriction may relate to different maternal adaptations to HF feeding between species.

The HF model used in the current study avoided the gestational and lactational difficulties encountered by other researchers into overnutrition. We experienced some problems with mating HF dams, but had no issues with gestation or labour or lactation (378); we had no neonatal mortality or cannibalism (379); our litters were of normal size and sex ratio. Yet these pups were

[3] Small birthweight offspring have also been observed in other species challenged by HF diets during gestation – for example, in pigs 376. Hoffman EC, Wangsness PJ, Hagen DR, Etherton TD. Fetuses of lean and obese swine in late gestation: body composition, plasma hormones and muscle development. Journal of animal science. 1983;57(3):609-20. and in guinea pigs 377. Pavey DE, Widdowson EM, Robinson MP. Body lipids of guinea pigs exposed to different dietary fats from mid-gestation to 3 months of age. II. The fatty acid composition of the lipids of liver, plasma, adipose tissue, muscle and red cell membranes at birth. Nutrition and metabolism. 1976;20(5):351-63.

reliably and significantly growth retarded at birth, and their subsequent weight gain during lactation and post-weaning was significantly different from Controls.

4.4.5 Growth during lactation

The pups of the MHF and PLHF groups were both born significantly lighter than CONT, however, by weaning (22 days later) these two groups had surpassed the Controls in weight gain and had become significantly heavier. This accelerated growth during lactation is typical of the catch-up growth seen in some other IUGR models, for example in undernutrition during pregnancy (110, 112, 113, 380). This is suggestive of a common mechanism of catch-up growth following either undernutrition or HF feeding during pregnancy, a common response to either extreme of nutrition.

Rats are an altricial species, born largely 'premature' in comparison to humans, with many organ systems undergoing rapid development after parturition. This means that lactation remains a 'sensitive period' in terms of programming, a time during which tissues or physiological functions are still malleable to environmental influences such as maternal diet (368), before becoming 'hard-wired'. For example, it is possible to produce a form of overnutrition isolated solely to the period of lactation. This can be done by reduction of litter size, and it will produce an obese phenotype (381).

The metabolic programming set in place by maternal HF feeding during pregnancy and/or lactation persisted after weaning.

4.4.6 Post-weaning growth

Throughout the post-weaning period (P22-160) the offspring of both MHF and PLHF dams continued their faster weight gain over the CONT offspring, irrespective of post-weaning diet. The increase in body weight of the offspring of the two maternal HF treatment groups over the Controls continued to expand as the animals aged, regardless of whether the post-weaning diet was Chow or HF (see Figure 4.6). DEXA body scanning showed that this weight gain was due to an increase in adiposity, an increase in adipose tissue as percentage of total body mass. At cull, the weights of the dissected retroperitoneal fat pad were also significantly different, confirming the elevated adiposity of the MHF and PLHF offspring over Controls, in both males and females. There was no significant difference between MHF and PLHF groups.

Although the maternal HF diet resulted in obese offspring regardless of postnatal nutrition, there was also a significant interaction between the effect of the maternal diet on postnatal diet. When CONT males were fed a post-weaning HF diet, they demonstrated a greater increase in diet-induced body fat accumulation compared to offspring of HF-fed mothers. It is possible that the MHF and PLHF offspring were better able to metabolically handle a postnatal HF diet. That is, these offspring were adaptively more suited to postnatal HF because of their formative intrauterine nutritional experience. These data are suggestive of a predictive adaptive response in these offspring (283)[4]. By contrast, this interaction was not seen in females: body fat accumulation in response to a postnatal HF diet was no different between the female CONT, MHF or PLHF groups. Thus, the adaptive response was gender specific.

The offspring of the two maternal HF treatments were phenotypically similar – both MHF and PLHF offspring were obese (irrespective of post-weaning nutrition), and were hyperinsulinaemic and hyperleptinemic compared to Controls. These data suggest that at least in rats, maternal body composition at the time of conception had no additive effect on offspring phenotypic outcome. It is possible that, although the MHF dams exhibited significantly greater diet-induced obesity at the time of mating, this may not have been great enough for synergistic effects to be observed. It could be challenging to test this, because excessive degrees of preconceptional obesity in rodents raise experimental problems. Firstly, marked obesity results in lack of normal estrus cycling (as observed in the present study) and thus reduced reproductive success, and secondly, marked obesity has also been known to result in lactational failure leading to high mortality in offspring (135, 137, 382).

There were a few obvious early differences between the MHR and PLHF offspring. Though both groups were small at birth, the PLHF pups were significantly smaller than the MHF pups. It appears the abrupt transition onto a HF diet may have affected the PLHF dams and the intrauterine environment of their pups to a degree that the MHF dams (raised on HF from weaning) did not experience. This difference in offspring body weight persisted through the lactation period - the MHF pups were still significantly heavier than the PLHF pups at weaning. However, this difference does not last long – an analysis of the post-weaning growth curves shows no difference between MHF *versus* PLHF, either among males or females. (Although see the male HF postnatal diet curves, where MHF offspring were beginning to gain significance *versus* the PLHF. It appears likely this significance would become established, had the animals

[4] An alternative hypothesis is that these male HF-exposed offspring may have reached a maximal threshold of body fat accumulation. However, the rats in the present study were still gaining weight when culled at age 180 days – please refer to the post-weaning growth curves.

lived longer.) When the offspring underwent DEXA scanning at 150 days of age there was no longer any significant difference in body weight between MHF and PLHF offspring, nor in degree of adiposity (% body fat).

The observation that a HF diet from weaning to conception did not confer phenotypic differences in offspring distinguishable from those who were fed the HF diet through pregnancy and lactation alone is intriguing and underscores the importance of nutrition throughout the lifetime of a reproductively active individual.

4.4.7 Energy intake: same caloric intake, but different adiposity

The rats on the HF diet gained weight by storing more fat in their adipose depots, even though their caloric intake per kg body weight was no different from their leaner Chow-fed siblings. This suggested they have a different energy balance, a different balance of energy intake *versus* expenditure. This could be through altered physical activity, which we did not measure. Or perhaps their metabolism was different, altered by the continuous high levels of fat ingested, so that fewer calories were burnt and more calories were stored. Other researchers have postulated similar conclusions regarding metabolic alteration (140, 349). West & York (1998) discussed the hyperphagia seen in some models of HF feeding, but argued that since weight gain still occurred without hyperphagia in other models (i.e., caloric intakes were not raised), then metabolic adjustments were likely.

In human studies, it appears that fat is not a strong satiety signal (383, 384) and this may be one reason why people tend to over-consume fatty foods. There could be an evolutionary advantage in this tendency, derived from an ancestry where food supplies were uneven and energy dense foods only occasionally available, so that the ability to store fat efficiently for times of scarcity enhanced survival. In modern Western societies scarcity is rarely a problem any longer, and excess storage of fat tends to expand the visceral depots ("central adiposity"), more than the subcutaneous depots. In humans different types of dietary fat are adipose depot site specific (385), with increased visceral adiposity in particular associated with increased risk of cardiovascular disease (386, 387).

It is of relevance that the protein contents of the two diets we utilised were closely similar. Several mechanisms are involved in regulating consumption (388), and levels of dietary protein alone have been shown to affect feeding behaviour in rats (389, 390). In pregnancy, protein stores are built up during the first two weeks and utilised for rapid fetal growth. Fat accumulated during

gestation is mobilised to fuel the energetic cost of lactation (391). Other signalling agents known to exert influence on food intake include glucose, insulin and leptin (392).

4.4.8 Onset of puberty

Exposure to a maternal HF diet (in both MHF and PLHF dams) lead to early pubertal onset in offspring, in both males and females. These findings are compatible with a previous report of early puberty following high fat nutrition during pregnancy (393). The maternal diet effect on MHF and PLHF offspring advanced the age of puberty to the same degree as did a post-weaning HF diet in CONT offspring. Intriguingly, the addition of a postnatal HF had no additive effect on age of pubertal onset. Therefore, nutritionally-induced acceleration of pubertal onset following HF exposure may not be limited to one distinct critical developmental window.

Developmental programming through early life exposure to HF altered both the metabolic phenotype (offspring had accelerated weight gain irrespective of postnatal diet, and were hyperinsulinaemic and hyperleptinemic in adulthood), and reproductive maturation. The effect of obesity is likely to be transgenerational. It is possible that there could also be a perpetuation and possibly a compounding of advanced pubertal onset in ensuing generations as these offspring will themselves be fatter at an earlier reproductive age.

Reproductive maturation in the MHF and PLHF offspring was not linked to increased body weight – indeed, these pups were significantly lighter than Controls when entering puberty. These lighter body weights were due to younger age of pubertal onset. (When same age pups are compared, MHF and PLHF offspring have already surpassed CONT in body weight – see Table 4.4 and Figure 4.6) Thus, body weight did not predict the age at puberty.

4.4.9 Plasma insulin and leptin concentrations in adulthood

We have shown in the present study that offspring from maternal HF treatment groups were normoglycemic, but hyperinsulinaemic and hyperleptinemic – and circulating hormone levels were increased even further by a postnatal diet of HF.

The normoglycemia is in contrast to other models where hyperglycemia has been induced in offspring by maternal diet alone (374). However, this tight homeostatic blood glucose regulation that we observed was only achieved by elevated insulin levels in the maternal HF offspring groups. (Maternal Diet Effect: CONT *vs* MHF & PLHF in males, $P<0.004$, CONT *vs* MHF in

females, P=0.023; Postnatal Diet Effect, Chow *versus* HF: P<0.001 in both males and females.) There were no statistical differences in insulin concentrations between MHF and PLHF groups. These raised plasma insulin levels are suggestive of insulin resistance, which is often linked with obesity in rats or with high fat feeding during development (140, 394). Obese humans also tend to exhibit insulin resistance, and insulin resistance is a precursor of diabetes mellitus (395).

Insulin resistance is also seen in pregnancy, where it is normal for pregnant women to develop some insulin resistance in late gestation. This is due to the normal increase in adipose tissue which is laid down in early pregnancy in order to supply energy and nutrients for fetal development, the bulk of which takes place in late gestation. Human babies are born fat relative to other mammals (338). Thus, an obese woman of reproductive years may already carry an on-going burden of insulin resistance, and should she then conceive, the further insulin resistance of pregnancy may tip her into gestational diabetes mellitus (GDM). GDM tends to produce a macrosomic baby, predisposed to obesity in later life and Type 2 Diabetes (T2DM). This can then become a transgenerational repeating pattern of morbidity, and such programming may be contributing to the increase in obesity and early onset diabetes currently seen in Western and developing societies (396).

Plasma leptin levels also revealed a significant programming effect due to maternal HF diet, which again was exacerbated by postnatal diet, with no statistical difference between MHF and PLHF offspring. Raised leptin levels are suggestive of leptin resistance. A maternal HF diet has been shown to result in offspring with leptin resistance at the level of the hypothalamus, leading to increased weight gain in adulthood (352). A neonatal leptin surge occurs in rodents at approximately neonatal Day 10, and it is hypothesised that this leptin surge is associated with maturation of neurons within hypothalamic appetite centres, rather than peripheral fat deposition or body weight gain (277, 397). Although we did not measure leptin repeatedly throughout the first 10 days of life, it is possible that reduction in leptin production or sensitivity that we observed in early neonatal life (P2) may significantly alter the establishment of a central appetite sensing pathway. Such a perturbation could have long-term repercussions on adult metabolic capabilities (123, 398).

The Insulin:leptin ratio is a marker of the functioning of the adipoinsular axis (235), representing something of the feedback balance between the two hormones. Our data demonstrated there was no significant difference between the maternal HF treatment groups *versus* Controls, in either males or females, suggesting that, although MHF and PLHF offspring were both

hyperinsulinemic and hyperleptinemic, the balance of the two adipoinsular hormones remained unchanged.

What is strikingly different, however, between male *versus* female, is the postnatal diet effect, which is very marked in the males ($P<0.001$ across all groups) while nonexistent in the females. HF-fed males in all groups had significantly reduced Insulin:leptin ratios, evidence that plasma leptin levels rose out of proportion to insulin levels in response to HF feeding. This may be a sign of leptin resistance within the males.[5] By contrast in females, while the postnatal HF diet also raised insulin and leptin levels, the balance between the two hormones was not altered.

These glucose, insulin and leptin data further indicate that preconceptional HF feeding of rat dams had little additive programming effect on offspring phenotype over HF feeding during pregnancy and lactation alone.

4.4.10 Summary

We have investigated the effect of feeding rat dams a HF diet under two conditions – during pre-conception and/or pregnancy and lactation, and the resultant programming of offspring coupled with the effect of postnatal diet. We have shown a maternal diet effect that is independent of postnatal diet, but exacerbated by postnatal HF feeding. This phenotype, programmed by HF during gestation and suckling, is growth retarded at birth, but goes on to gain excessive weight, to enter puberty early, and is insulin and leptin resistant in adulthood. Interestingly, the programmed offspring exhibited increased adiposity despite a caloric intake which is no different from Controls. The mechanism of this altered metabolism and energy balance is uncertain and merits further investigation. The maternal diet effect is due to HF feeding during pregnancy and lactation, and appears to be less dependent on preconception diet.

This model has close parallels with women who are obese during pregnancy: they also are likely to be hyperinsulinaemic, and their babies are more prone to obesity in later life with insulin resistance and type 2 diabetes. Thus obesity becomes a self propagating cycle of malprogramming, which may well contribute to the increase in diabetes seen in developed countries. These data suggest that obese women planning to have a baby would be advised to lose

[5] An alternative hypothesis is that the postnatal diet effect seen in the male Insulin:leptin ratio reflects the increased adiposity of males over females. However, postnatal diet effects on adiposity (% Body Fat changes) are significantly variable within males and much less so within females – see Figure 4.8C – and do not closely match the stable differences in Insulin:leptin ratio seen between genders.

weight before becoming pregnant, in order to afford the developing fetus and neonate the best programming advantage in life.

Chapter 5: Gene Expression

5.1 Introduction

Maternal nutritional adversity is associated with increased risk of obesity and metabolic compromise. Previous studies have suggested that not only do offspring develop peripheral insulin resistance (24, 294, 394, 399) but that the relationship between the pancreas and adipose tissue, the adipoinsular axis, may be compromised in offspring after early life nutritional adversity (114, 234, 236, 238). In early studies, Dutch Hunger Winter data revealed an association between exposure to gestational undernutrition and increased adiposity and glucose intolerance in later life (24, 400), effects which were dependent upon the timing of the period of famine.

However, the most common form of adverse maternal nutrition in developed societies comes not from famine or undernutrition but via the burden of adiposity that many mothers bring to conception and pregnancy (401). Maternal obesity is now the most common clinical risk factor in obstetrics (402) with elevated incidence of complications such as miscarriage, operative delivery and pre-eclampsia (403). In a rat model, it has been shown that maternal obesity alone can alter neurons of the hypothalamic appetite regulatory centre and leptin concentrations in neonates, and that such offspring go on to exhibit increased adiposity and insulin resistance (134).

It is thought that an adverse maternal nutrition may affect the development of the fetus by altering expression of genes in susceptible cells (404). Such adjustments may then persist into adult life, permanently modifying phenotype and metabolic profile. For example, Pdx1 is a key gene in the early development of the fetal pancreas in the rat. Intrauterine growth restricted (IUGR) rat fetuses showed pancreatic changes leading to diabetes in adulthood, and epigenetic modification in Pdx1 gene expression, which resulted in silencing of Pdx1 transcription in adult offspring (107). Other factors regulating islet vascularisation, β-cell proliferation and insulin secretion may also be subject to altered gene expression (405), including the ATP-sensitive potassium channel (406). Moreover, gene expression levels in other tissues were also modified in IUGR offspring, such as the glucose transporter GLUT4 in skeletal muscle, thereby contributing to insulin resistance (407, 408).

In the relationship between the pancreatic β-cell and white adipose tissue, the adipoinsular axis, insulin stimulates adipocytes to promote adipogenesis and leptin production, and circulating

leptin in turn restrains insulin release from the β-cell (234). In our model, offspring of high fat (HF)-fed mothers exhibited both hyperinsulinemia and hyperleptinemia, suggesting a perturbation of the adipoinsular signalling pathway, with a failure of leptin-mediated restraint on pancreatic insulin release (Chapter 1). We hypothesised therefore that key genes regulating leptin and insulin signalling in the pancreatic β-cell were significantly impaired in these offspring.

The glucose-stimulated insulin secretion pathway in the β-cell has been well characterised (409). Insulin secretion is regulated in accordance with plasma glucose levels, and modulated by hormonal and autonomic input, including autocrine feedback via the insulin receptor (IR) on the β-cell membrane (410). A key link between the glucose sensing regulatory factors and insulin granule exocytosis is the ATP-sensitive potassium channel (411). The activity of this ion channel is directly regulated by leptin as a means of restraining insulin secretion. Leptin has other less direct actions as it suppresses transcription of the preproinsulin and insulin promoter genes, via activation of JAK/STAT pathways. Moreover, the JAK/STAT signalling pathway is regulated by the Suppressor of cytokine signalling 3 protein (SOCS3), another product of leptin activation, and this has been proposed as a mechanism of leptin resistance in the hypothalamus (412, 413).

Some key genes in the insulin- and leptin-signalling pathways have been investigated before, in various tissues. Leptin receptor (ObRb) expression levels were down-regulated with leptin resistance in the hypothalamus (414-416) and skeletal muscle (417). The role of SOCS3 in leptin resistance has been demonstrated not only in the hypothalamus, but also in adipocytes (418, 419), in skeletal muscle (417), and in an ovarian cell line (420, 421). In the pancreas, leptin suppresses transcription of the preproinsulin gene (208, 422), the rat insulin 1 gene promoter (423), the protein phosphatase 1 gene (209), and activates transcription of the SOCS3 gene (209, 216). (See Figure 5.1).

Signalling pathways downstream of the leptin receptor are illustrated in Figure 5.1. It has been asserted that the IRS / PI3K / PIP_3 signalling pathway is the main mediator of leptin's inhibitory action on insulin secretion (209, 424). However, the JAK/STAT pathway also restrains insulin synthesis, through inhibition of insulin gene transcription. Disruption of either (or both) of these pathways could be the source of leptin insensitivity in the β-cell.

Using pancreata from offspring of our two maternal HF treatment cohorts (MHF dams: raised on HF nutrition from weaning and through pregnancy and lactation; PLHF dams: raised on standard Chow and transferred to a HF diet only during pregnancy and lactation), we investigated the mRNA levels of genes directly involved in glucose stimulated insulin secretion, namely IR, IRS1

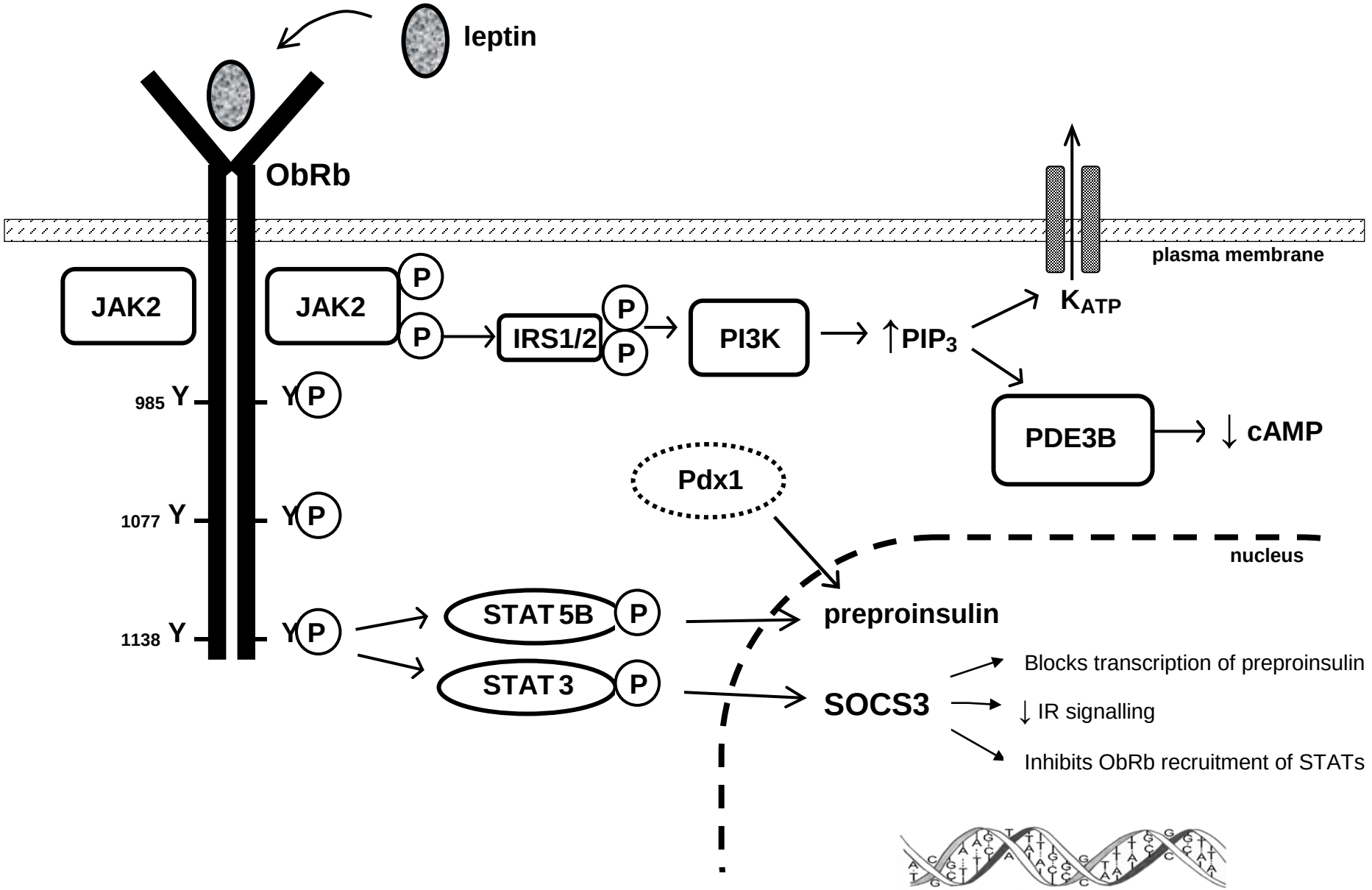
& IRS2, the Kir6.2 subunit of the K_{ATP} channel, and Pdx1, and determined leptin signalling by investigating mRNA levels of ObRa, ObRb, SOCS3, PI3K, and PDE3B. Leptin mRNA levels were also investigated, because although leptin is not synthesised by the β-cell itself, it has been found in δ-cells within the Islets of Langerhans (238).

We hypothesised that the compromised metabolic phenotype of male offspring born to mothers fed HF nutrition would be reflected in altered mRNA levels of transcription factors and second messenger systems regulating leptin signalling pathways within pancreatic β-cells. By examining genes in multiple signalling pathways we hoped to elucidate the mechanism/s underlying leptin resistance in these animals. Correlation analyses were used to look for relationships between mRNA levels of the different genes, and we hypothesised that correlations would be consistent with any alterations in leptin signalling pathways.

Figure 5.1: **Intracellular signalling pathways regulated by ObRb.** (see following page)

Diagram represents the leptin receptor long form (ObRb) on the β-cell membrane, with two downstream signalling pathways illustrated: the IRS / PI3K / PIP3 signalling pathway regulating the K_{ATP} channel (upper part of diagram), and the JAK/STAT pathway regulating gene transcription within the nucleus via SOCS3 (lower part of diagram). Diagram adapted from Becker, 2009.

Abbreviations are: ObRb = leptin receptor long isoform; JAK2 = Janus kinase 2; Ⓟ = phosphorylation; IRS1 = insulin receptor substrate 1; IRS2 = insulin receptor substrate 2; PI3K = phosphoinositide 3-kinase; PIP_3 = phosphoinositide$_{3,4,5}$ triphosphate; K_{ATP} = ATP-sensitive potassium channel (ATP = adenosine triphosphate; PDE3B = phosphodiesterase 3B; cAMP = cyclic adenosine monophosphate; Y = tyrosine residue on cytosolic portion of ObRb; Pdx1 = pancreatic and duodenal homeobox 1; STAT 5B = signal transducer and activator of transcription 5B; STAT3 = signal transducer and activator of transcription 3; SOCS3 = suppressor of cytosine signalling 3; IR = insulin receptor.


leptin
ObRb
plasma membrane
JAK2
JAK2
P
P
IRS1/2
P
P
PI3K
↑PIP3
KATP
985 Y
Y P
PDE3B
↓ cAMP
Pdx1
1077 Y
Y P
nucleus
1138 Y
Y P
STAT 5B
P
preproinsulin
STAT 3
P
SOCS3
Blocks transcription of preproinsulin
↓ IR signalling
Inhibits ObRb recruitment of STATs

5.2 Materials and Methods

5.2.1 Animals

In this study, we used an established model of developmental programming via maternal nutrient manipulation (296, 425). The study design utilised and the endpoint indices measured were described fully in Chapters 2 & 4, but will be reiterated here in brief. The pancreatic tissue used here is from the animals studied in Chapter 4, the offspring of the maternal HF treatment groups. All animal work was approved by the Animal Ethics Committee of the University of Auckland.

Wistar rats were acquired at weaning (22 days old) and housed in the animal facility under standard 25°C/humidity-controlled conditions – 2 per cage, 12:12 hour light dark cycle, with free access to water. Females were weight matched and randomly assigned to either: a) *ad libitum* standard rat chow (Diet 2018, Harlan Teklad, Oxon, n=16); or b) *ad libitum* high-fat diet (45% kcals as fat, D12451, Research Diets, NJ, USA, n=8). Males were fed *ad libitum* chow (n=12). These nutritional regimes continued unchanged until mating occurred; body weights were recorded twice weekly.

At age 110±5 days the animals were time mated. Estrous was determined using an FC40 Estrous Cycle Monitor (Fine Science Tools, USA), the male was introduced to the female overnight, and mating was confirmed under light microscopy next morning by examining vaginal lavage fluid for the presence of sperm or mucosal plug. Once pregnant the females were caged individually with food intake and weight measured daily; the male rats were sacrificed.

Pregnant females were randomised into three maternal dietary groups:
1) **Controls (CONT)**: Females that had been raised on standard rat chow *ad libitum* since weaning, and then maintained on the same chow diet throughout pregnancy and lactation;
2) **Maternal High Fat (MHF)** group: Females raised on the *ad libitum* high-fat (HF) diet since weaning, and continued on the same HF diet throughout pregnancy and lactation; and
3) **Pregnancy and Lactation High Fat (PLHF)** group: Females raised on *ad libitum* chow since weaning, then transferred to *ad libitum* HF during pregnancy and lactation.

At birth pups were weighed and measured for length. Litter size was standardised to eight pups (by preference 4 male and 4 female) to ensure comparable nutrition. Throughout the lactation period dams were measured daily for body weight and food intake. Pups were weighed on days 1 and 2, then every second day thereafter, until weaning at day 22. At weaning, genders were

separated; pups were weight matched within their maternal dietary groups, and accommodated two per cage per litter per maternal nutritional treatment.

The current study into pancreatic gene expression only investigated male adult offspring: females were not studied because of the possible complicating factor of estrous effects. Moreover, only those males raised on post-weaning Chow were utilised: this was for logistical reasons, and because the most stark phenotypic contrast was evident between the Chow-fed cohorts. It also meant that the only experimental variables were in the maternal diet.

5.2.2 Plasma and Tissue Collection

At age 180 days animals were fasted overnight, were anaesthetised by pentobarbitone injection (60mg/kg, IP) and once pain reflexes were absent, killed by decapitation. Blood was collected into heparinised Vacutainer tubes and stored on ice until centrifuged to yield plasma for analysis. (Centrifuged: 1500 x *g* at 4 ºC for 15 minutes, then stored at -20 ºC.) Whole pancreas was harvested immediately, snap frozen in liquid nitrogen and stored at -80°C.

Plasma Analyses Commercial rat-specific ELISA kits were used to determine plasma insulin and plasma leptin levels (Crystal Chem, 90060 and 90040 respectively, Uppsala, Sweden), as described in Chapter 2.

5.2.3 Gene Expression

Following whole pancreas tissue disruption, total RNA extraction was achieved using Betamercaptoethanol and Qiagen Mini-Prep kits (Catalogue number 80204) according to the manufacturer's instructions. All RNA had $A_{260/280}$ >1.9 and $A_{260/230}$ >2.0, and was intact when visualised by gel electrophoresis. cDNA was synthesised from RNA (5µg) by reverse transcription PCR (using MMLV-RT from Invitrogen, CA, USA), and then used as a template for quantitative (q) PCR, to explore relative levels of gene expression. qPCR was performed in 15µl reaction volumes using EXPRESS SYBR GreenER (Invitrogen), and fluorescence was measured and quantified using an ABI-7900HT Ver.2.3 Sequence Detection System (Applied Bio Systems, CA, USA). The qPCR thermal profile for amplification of all genes was: melt at 95°C for 15secs, followed by anneal/extend at 60°C for 1min, for 40 cycles.

A Standard Curve was generated using 6-fold serial 1-in-5 dilutions of a stock made from 3ul of each sample's cDNA . The Ct values were plotted against the log of the template amount, to

produce a straight line. This Standard Curve was then used to calculate the amount of template in the experimental samples. A trial was conducted with each primer using a limited range of experimental samples, to determine the optimal concentration of sample cDNA to use. Typically a dilution of 1:15 cDNA proved satisfactory, placing the majority of the C_t values in the mid range of the Standard Curve. All samples were then assayed in triplicate: individual readings were omitted if Ct values differed by >0.5 or if dissociation curves differed from those in the Standard Plot. Only dissociation curves exhibiting a single PCR product were accepted. All qPCR results were normalised against the geometric mean of Cyclophilin x HPRT, according to the GeNorm method of Vandesompele *et al.* (2002) (426).

5.2.4 Primer Design

Primers were obtained as either commercially available products, or as sequences published in the literature, or designed *de novo*. (Table 5.1) New primers were designed using NCBI 'Primer-BLAST' with the following conditions: primers were 18-24 nucleotides in length; forward and reverse primers had similar melting temperatures (around 65°C, about 5° more than the PCR annealing temperature (290)); contained approximately 50% GC content; primers spanned exon-exon boundaries where possible; mononuclear repeats of 4 or more bases were avoided; and amplicons were 100-200bp long.

5.2.5 Data Presentation

The mean mRNA levels of Control offspring have been standardised to 100 in all bar graphs, with MHF and PLHF offspring levels represented as comparative fold-changes. The units of gene expression generated by quantitative real-time PCR are relative rather than absolute, thus fold changes relative to CONT are a useful measure of difference when comparing gene with gene.

5.2.6 Statistical Analyses

Data was analysed using the SigmaStat 3.1 statistical package (SYSTAT Software Inc, IL, USA). Differences between mean mRNA levels in offspring groups were determined by One Way ANOVA, with maternal diet as factor. Statistical significance was accepted at the $P < 0.05$ level. All data are presented as means ± SEM unless otherwise stated.

Correlations were graphed and analysed using GraphPad Prism version 5.03 from GraphPad Software Inc, San Diego, CA 92121, USA.

Table 5.1: **Sequences of Forward and Reverse Primers**

	Entrez Gene ID	Forward Primer	Reverse Primer
K^+ Channel (Kir6.2)	83535	5'-GAA GGA GGC AAA TGA TTG GA-3'	5'-AGT GTC CCC CAG ACA AAG TG-3'
PDE3B	29516	5'-GAC CGT CGT TGC CTT GTA TT-3'	5'-CGA TCG CCT TTC TCT ACT GG-3'
SOCS3	89829	5'-TTC TTT ACC ACC GAC GGA AC-3'	5'-CGT TGA CAG TCT TCC GAC AA-3'
Insulin1	24505	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00373303 (Qiagen)	
Insulin2	24506	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00177380 (Qiagen)	
Insulin Receptor	24954	5'-ATC CGT CGC TCC TAT GCT CT-3'	5'-TCG TGA GGT TGT GCT TGT TC-3'
Leptin	25608	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00374801 (Qiagen)	
IRS1	25467	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT01623349 (Qiagen)	
IRS2	29376	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00190960 (Qiagen)	
PI3K	25513	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00184485 (Qiagen)	
ObRa from ref [55]	24536 *	5'-TGA TAT CGC CAA ACA GCA AA-3'	5'-AGT GTC CGC TCT CTT TTG GA-3'
ObRb	24536 *	5'-AAA GCC TGA AAC ATT TGA GCA TC-3'	5'-CCA GAA GAA GAG GAC CAA ATA TCA C-3'
Pdx1	29535	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00405328 (Qiagen)	
STAT3	25125	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00183512 (Qiagen)	
STAT5B	25126	Commercially prepared primer, QuantiTect Primer Assay, Cat.# QT00192024 (Qiagen)	
Cyclophilin	25518	5'-TTG GGT CGC GTC TGC TTC GA-3'	5'-GCC AGG ACC TGT ATG CTT CA-3'
HPRT	24465	5'-AGT CCC AGC GTC GTG ATT AG-3'	5'-CCC CCT TCA GCA CAC AGA-3'

* ObRa and ObRb are splice isoforms of the same singular leptin receptor gene

5.3 Results

The full phenotypic description of dams and offspring were presented in Chapter 4, but details of particular pertinence to the current chapter are reiterated in brief below.

5.3.1 Maternal phenotype

At the time of mating, females raised on a pre-conception HF diet (MHF dams) had significantly increased body weights and percentage body fat compared to females raised on Chow (CONT and PLHF dams), see Table 4.1. There was no significant difference in weight gain during pregnancy between maternal groups, see Figure 4.3.

At weaning, (P22), both MHF and PLHF dams were hyperinsulinemic compared to CONT dams, with PLHF dams recording significantly higher insulin levels than MHF dams. There was no difference between maternal groups in plasma leptin levels. See Table 4.6.

5.3.2 Offspring phenotype

Please refer to Chapter 4 for details. In brief at the end of the study (P180) adult male offspring of both MHF and PLHF dams were obese, hyperinsulinemic and hyperleptinemic, compared to CONT offspring (Figures 4.8A and 4.9A, C). There was no difference between groups in plasma glucose levels (Table 4.7). In all these measures, there was no significant difference between the MHF *versus* PLHF offspring.

5.3.3 Pancreatic mRNA levels

5.3.3.1 Genes associated with insulin action and synthesis

Both pre-proinsulin genes found in the rat pancreas, Ins1 and Ins2 were significantly elevated in both PLHF and MHF offspring compared to Controls, although the difference in MHF Ins1 mRNA levels did not reach statistical significance (MHF *vs* CONT: P=0.053; Figure 5.2A, B). Ins1 and Ins2 mRNA levels were highly positively correlated with each other, in all three groups (Figure 5.2C). Interestingly, there was no correlation between plasma insulin concentrations and mRNA levels of either Ins1 or Ins2 genes (Ins1 *vs* plasma Insulin: CONT, $R^2 = 0.0009$, $P = 0.94$; MHF, $R^2 = 0.20$, $P = 0.32$; PLHF, $R^2 = 0.009$, $P = 0.77$; Ins2 *vs* plasma Insulin: CONT, $R^2 = 0.005$, $P = 0.87$; MHF, $R^2 = 0.08$, $P = 0.53$; PLHF, $R^2 = 0.004$, $P = 0.83$).

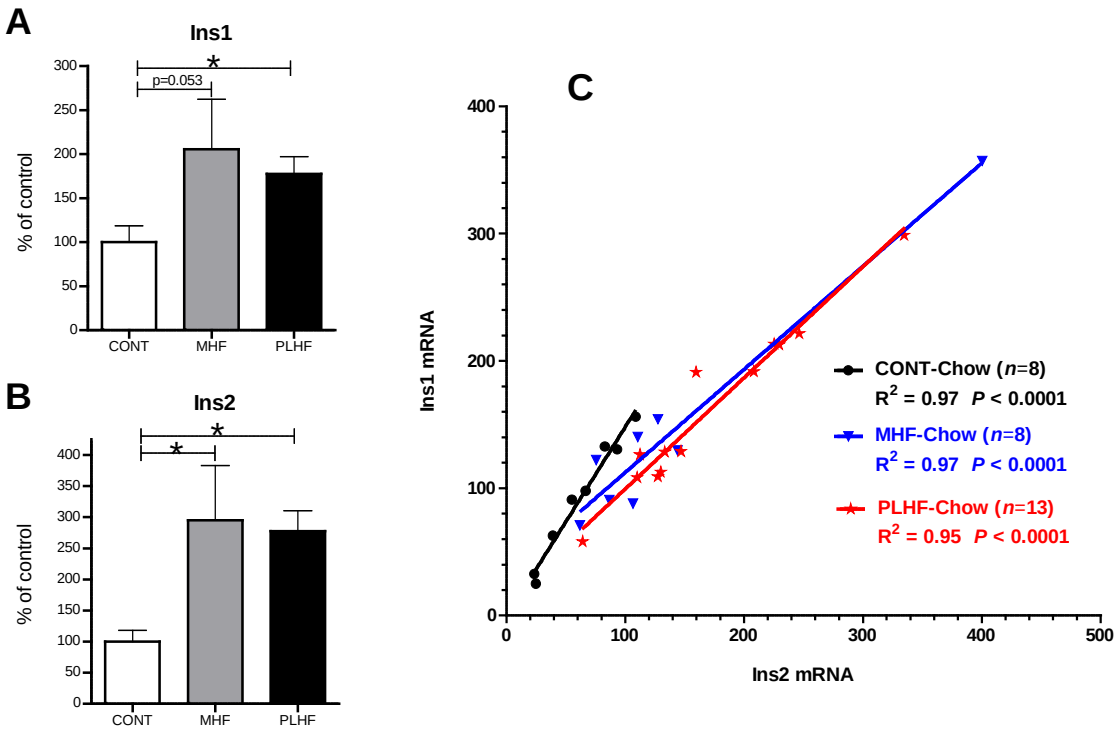


Figure 5.2: Relative pancreatic expression levels of Ins1 (n = 9-13) (A) and Ins2 (n = 8-13) (B) mRNA and correlations between pancreatic Ins1 and Ins2 expression (C) in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100. * $P < 0.05$.

Interestingly, Pdx1 mRNA levels were significantly different between MHF *versus* PLHF offspring, while neither MHF or PLHF were statistically different from controls (Figure 5.3).

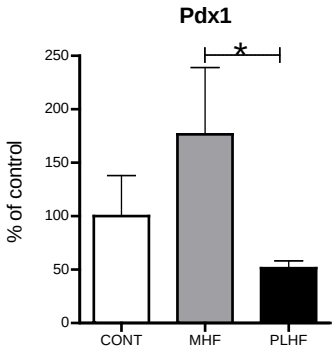


Figure 5.3: Relative pancreatic expression levels of Pdx1 mRNA in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100. n = 8-12. * $P < 0.05$.

Pdx1 mRNA correlated more often with other mRNAs than any other single gene within this study; moreover, the pattern of Pdx1 correlations was very different between the MHF *versus* PLHF groups. Pdx1 mRNA tended to positively correlate with both plasma insulin and plasma leptin (P = 0.07 and 0.06 respectively), and was positively correlated with SOCS3, IRS1 (Table 5.2) in CONT and MHF offspring. Pdx1 mRNA was negatively correlated with PI3K in Control offspring only (Table 5.2). In MHF offspring, Pdx-1 was also positively correlative with ObRb mRNA (Table 5.2). By contrast, the PLHF offspring had their own distinct pattern of correlations, demonstrating significant positive correlations between Pdx1 mRNA and STAT3, Ins1&2, and IR (Table 5.2). These relationships were not observed in CONT or MHF offspring. Of note, Pdx1 correlated strongly with K^+ channel mRNA in all three groups.

Table 5.2: **Correlations between Pdx1 mRNA levels and other genes and measures**

	CONT	MHF	PLHF
Pdx1 *vs* plasma leptin	R^2 = 0.47, P = 0.06	R^2 = 0.23, P = 0.23	R^2 = 0.003, P = 0.84 †
Pdx1 *vs* plasma insulin	R^2 = 0.45, P = 0.07	R^2 = 0.11, P = 0.47	R^2 = 0.09, P = 0.34
Pdx1 *vs* ObRb	R^2 = 0.05, P = 0.79 †	R^2 = 0.72, P = 0.007	R^2 = 0.02, P = 0.68
Pdx1 *vs* SOCS3	R^2 = 0.78, P = 0.009	R^2 = 0.91, P = 0.0003	R^2 = 0.07, P = 0.40 †
Pdx1 *vs* STAT3	R^2 = 0.06, P = 0.55 †	R^2<0.0001, P = 0.99	R^2 = 0.39, P = 0.03
Pdx1 *vs* Ins1	R^2 = 0.02, P = 0.72 †	R^2 = 0.02, P = 0.73 †	R^2 = 0.55, P = 0.006
Pdx1 *vs* Ins2	R^2 = 0.002, P = 0.91 †	R^2 = 0.04, P = 0.62 †	R^2 = 0.65, P = 0.001
Pdx1 *vs* IR	R^2 = 0.17, P = 0.36 †	R^2 = 0.23, P = 0.23	R^2 = 0.57, P = 0.005
Pdx1 *vs* IRS1	R^2 = 0.81, P = 0.006	R^2 = 0.54, P = 0.04	R^2 = 0.05, P = 0.46
Pdx1 *vs* IRS2	R^2 = 0.24, P = 0.26	R^2 = 0.53, P = 0.04	R^2 = 0.09, P = 0.33
Pdx1 *vs* PI3K	R^2 = 0.57, P = 0.049 †	R^2 = 0.08, P = 0.49	R^2 = 0.02, P = 0.70
Pdx1 *vs* PDE3B	R^2 = 0.05, P = 0.60 †	R^2 = 0.10, P = 0.45 †	R^2 = <0.0001, P = 0.99
Pdx1 *vs* K^+ channel	R^2 = 0.97, P = 0.0003	R^2 = 0.94, P<0.0001	R^2 = 0.78, P = 0.0003
Pdx1 *vs* Leptin mRNA	R^2 = 0.003, P = 0.93	R^2 = 0.02, P = 0.75	R^2 = 0.01, P = 0.73 †

† = negative correlation. All other unmarked correlations are positive.
(n = 5-12, significant correlations are shaded grey.)

Although insulin receptor (IR) mRNA levels were similar between groups (Figure 5.4A), IR demonstrated a significant positive correlation with both Ins1 and Ins2, (IR *vs* Ins1: CONT, R^2 = 0.30, P = 0.20; MHF, R^2 = 0.01, P = 0.78; PLHF, R^2 = 0.28, P = 0.06. IR *vs* Ins2: CONT, R^2 = 0.24, P = 0.26; MHF, R^2 = 0.08, P = 0.45; PLHF, R^2 = 0.37, P = 0.03 – see Figures 5.4B, C), but only in the PLHF offspring. IR mRNA demonstrated no significant correlation with IRS1 in any of the groups, but correlated positively with IRS2 in the PLHF group only (IR *vs* IRS2: CONT, R^2 = 0.40, P = 0.18; MHF, R^2 = 0.15, P = 0.30; PLHF, R^2 = 0.43, P = 0.02 – see Figure 5.4D).

Other IR correlations were with SOCS3 in CONT (see Table 5.4), and PI3K in MHF and PLHF (see PI3K results).

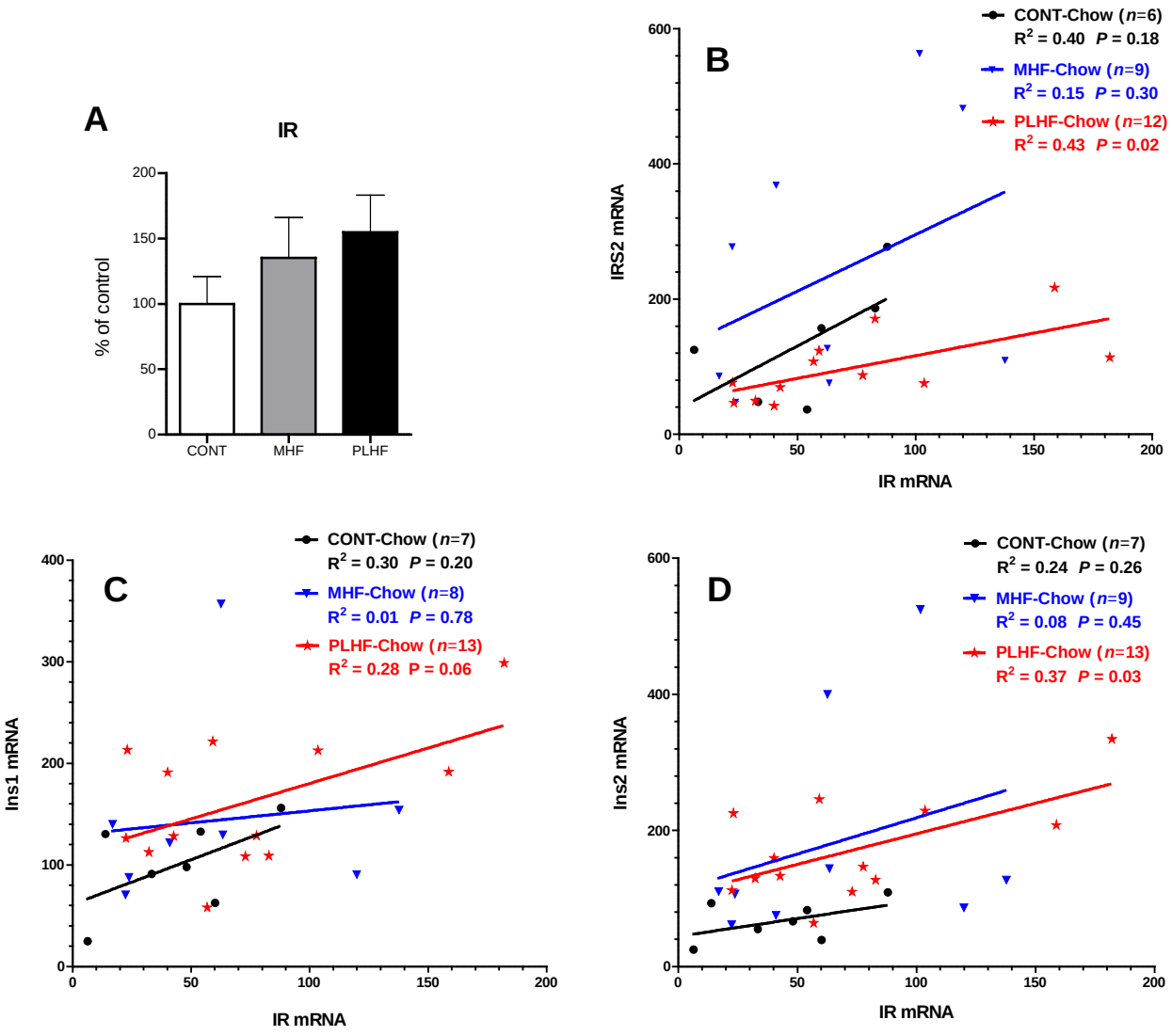


Figure 5.4: (A) Relative pancreatic expression of insulin receptor (IR) mRNA (n = 8-13); (B) correlations between pancreatic IR and insulin receptor substrate 1 (IRS1) mRNA; (C) correlations between pancreatic IR and insulin1 (Ins1) mRNA and (D) correlations between pancreatic IR and Ins2 mRNA in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100.

Pancreatic IRS1 mRNA levels in MHF offspring were significantly higher compared to both CONT and PLHF offspring. The highest IRS2 mRNA levels were demonstrated in the MHF group, although this difference was significant only compared to the PLHF offspring and not compared to CONT (Figure 5.5). IRS1 mRNA and IRS2 mRNA were positively correlated in MHF and PLHF groups, but not in Controls (Figure 5.5). (IRS1 *vs* IRS2: CONT, $R^2 = 0.44$, $P = 0.10$; MHF, $R^2 = 0.99$, $P < 0.0001$; PLHF, $R^2 = 0.72$, $P = 0.0005$).

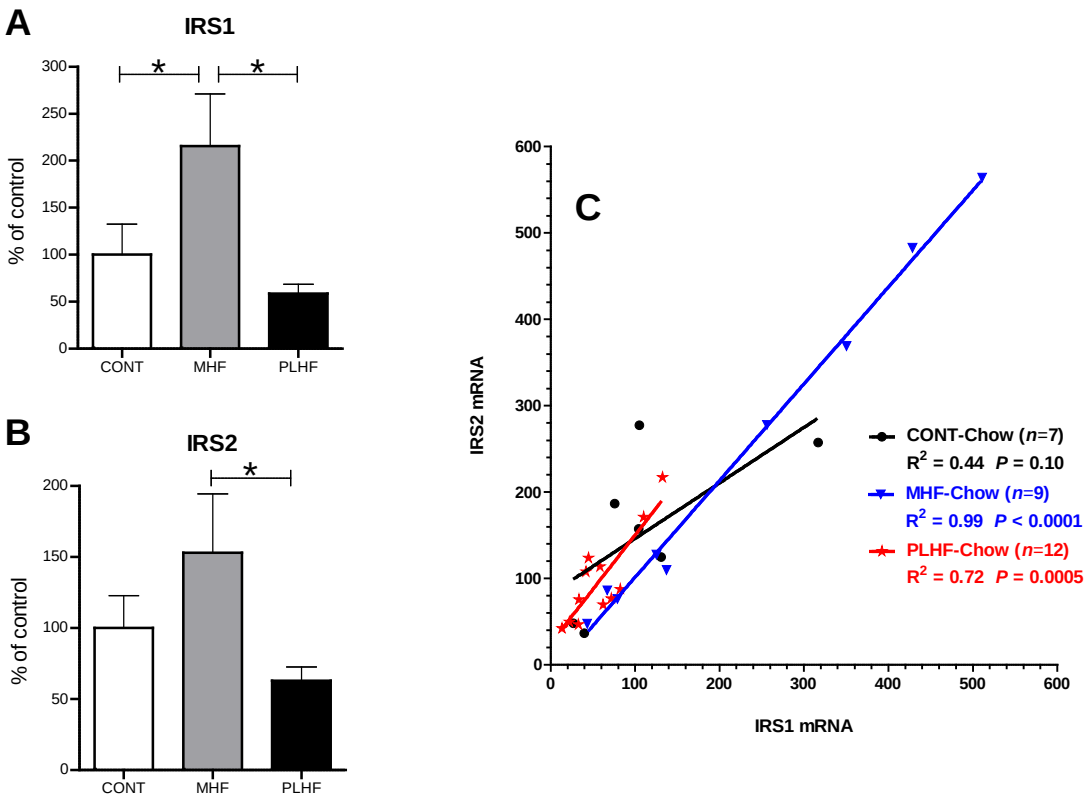


Figure 5.5: (A) Relative pancreatic expression of insulin receptor substrate 1 (IRS1) mRNA (n = 8-12); (B) Pancreatic expression of insulin receptor substrate 2 (IRS2) mRNA (n = 7-12) and (C) correlations between pancreatic IRS1 and IRS2 mRNA in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100. * $P < 0.05$.

Both IRS1 and IRS2 were positively correlated with SOCS3 mRNA in CONT and MHF groups; both positively correlated with PI3K in the PLHF animals; both positively correlated with Pdx1 in MHF offspring (Table 5.3). IRS1 mRNA and IRS2 mRNA were positively correlated with ObRb mRNA in MHF, but not in CONT or PLHF offspring (Table 5.3).

Table 5.3: **Correlations between IRS1 and IRS2 mRNA *versus* other genes**

	CONT	MHF	PLHF
IRS1 *vs* SOCS3	$R^2 = 0.84$, $P = 0.01$	$R^2 = 0.58$, $P = 0.02$	$R^2 = 0.04$, $P = 0.51$
IRS2 *vs* SOCS3	$R^2 = 0.98$, $P = 0.002$	$R^2 = 0.58$, $P = 0.02$	$R^2 = 0.15$, $P = 0.21$
IRS1 *vs* PI3K	$R^2 = 0.29$, $P = 0.22$ †	$R^2 = 0.07$, $P = 0.49$	$R^2 = 0.44$, $P = 0.02$
IRS2 *vs* PI3K	$R^2 = 0.01$, $P = 0.81$	$R^2 = 0.05$, $P = 0.57$	$R^2 = 0.72$, $P = 0.0005$
IRS1 *vs* Pdx1	$R^2 = 0.81$, $P = 0.006$	$R^2 = 0.54$, $P = 0.04$	$R^2 = 0.05$, $P = 0.46$
IRS2 *vs* Pdx1	$R^2 = 0.24$, $P = 0.26$	$R^2 = 0.53$, $P = 0.04$	$R^2 = 0.09$, $P = 0.33$
IRS1 *vs* ObRb	$R^2 = 0.67$, $P = 0.09$	$R^2 = 0.72$, $P = 0.008$	$R^2 = 0.23$, $P = 0.19$
IRS2 *vs* ObRb	$R^2 = 0.58$, $P = 0.24$	$R^2 = 0.70$, $P = 0.01$	$R^2 = 0.06$, $P = 0.53$

(n = 5-13, significant correlations are shaded grey.)
† = negative correlation. All other unmarked correlations are positive.

Pancreatic PI3K mRNA levels were lower in MHF and PLHF offspring compared to Controls but only differences in PLHF offspring were significant (Figure 5.6).

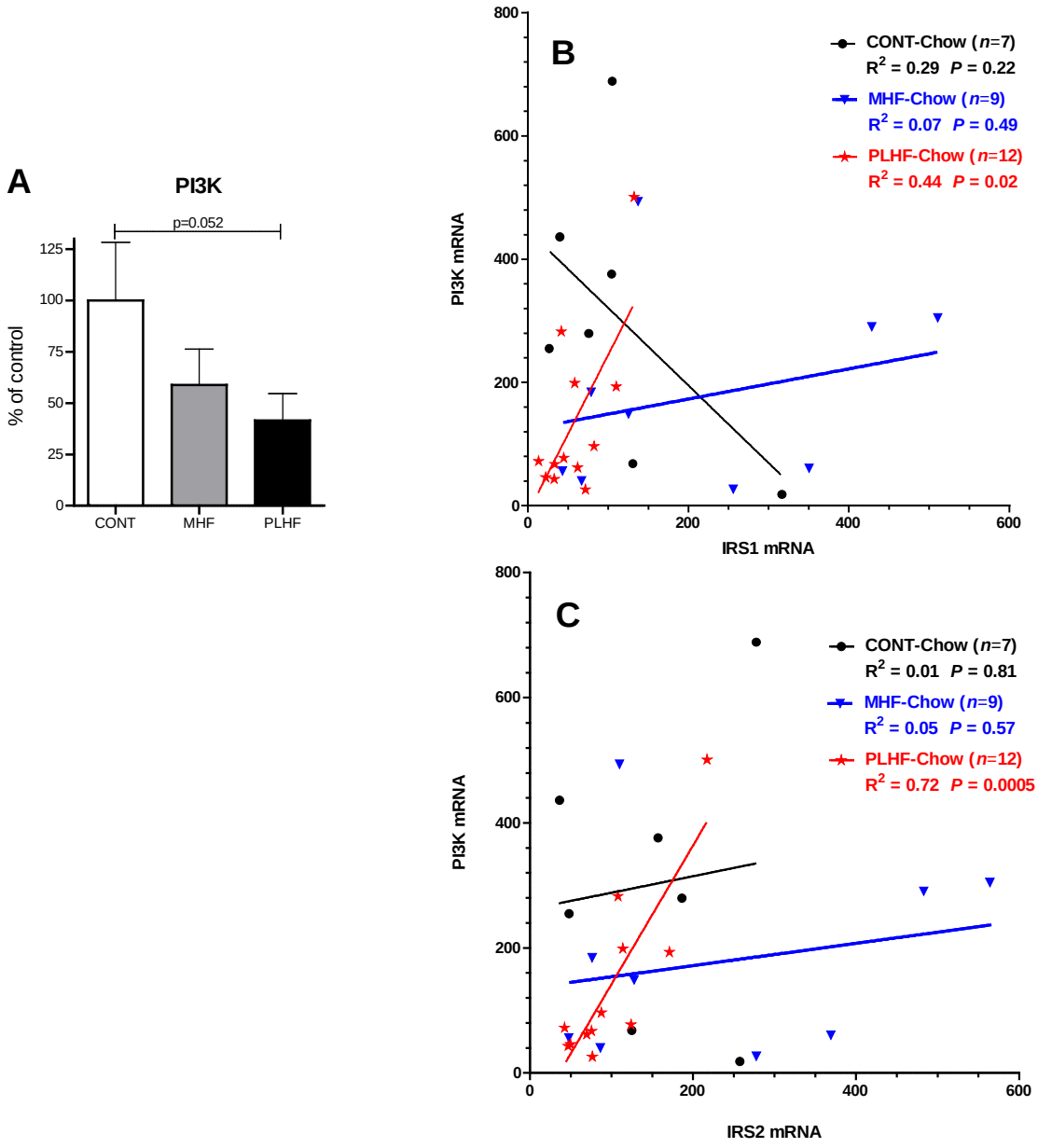


Figure 5.6: (A) Relative pancreatic expression of phosphoinositide 3-kinase (PI3K) mRNA (n = 8-12); (B) correlations between pancreatic insulin receptor substrate 1 (IRS1) and PI3K mRNA and (C) correlations between pancreatic insulin receptor substrate 2 (IRS2) and IR3K mRNA in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100.

Interestingly, PI3K mRNA levels also showed a positive correlation with IRS1 and IRS2 mRNA, only in PLHF offspring (see Table 5.3). CONT offspring demonstrated a negative correlation between PI3K and IRS1, but this relationship was positive in both MHF and PLHF offspring (Figure 5.6B). In contrast the relationship between PI3K and IRS2 was positive in all three

groups (Figure 5.6C). PI3K mRNA was significantly correlated with IR mRNA in MHF and PLHF offspring, and tended to correlate in Control offspring (PI3K *vs* IR: CONT, $R^2 = 0.61$, $P = 0.07$; MHF, $R^2 = 0.93$, $P < 0.0001$; PLHF, $R^2 = 0.46$, $P = 0.02$).

Pancreatic PDE3B mRNA levels were similar between groups (Figure 5.7A). Pancreatic K^+ channel mRNA levels were highest in the MHF offspring, although were only significantly different from PLHF offspring (Figure 5.7B).

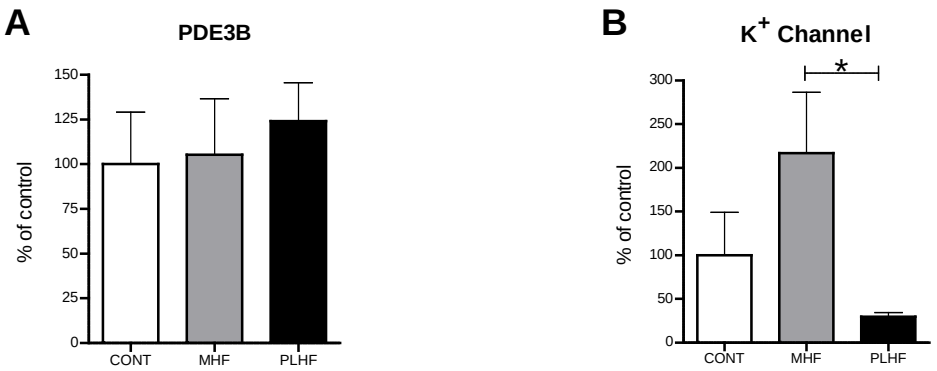


Figure 5.7: (A) Relative pancreatic expression of phosphodiesterase 3B (PDE3B) mRNA (n = 9-11) and (B) relative pancreatic expression of K^+ channel mRNA (n = 6-11) in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100. * $P < 0.05$.

K^+ channel mRNA levels showed a positive correlation with SOCS3 in CONT and MHF offspring (K^+ channel *vs* SOCS3: CONT, $R^2 = 0.82$, $P = 0.03$; MHF, $R^2 = 0.93$, $P < 0.0001$; PLHF, $R^2 = 0.002$, $P = 0.90$). K^+ channel was significantly correlated with ObRb mRNA in MHF offspring, and showed a trend toward correlation in PLHF, but not CONT offspring (K^+ channel *vs* ObRb: CONT, $R^2 = 0.07$, $P = 0.83$; MHF, $R^2 = 0.74$, $P = 0.006$; PLHF, $R^2 = 0.42$, $P = 0.08$).

5.3.3.2 Genes associated with leptin action and signalling

ObRb mRNA levels and the mRNA of its downstream signalling factor SOCS3 were highest in MHF offspring compared to both Control and PLHF (Figure 5.8).

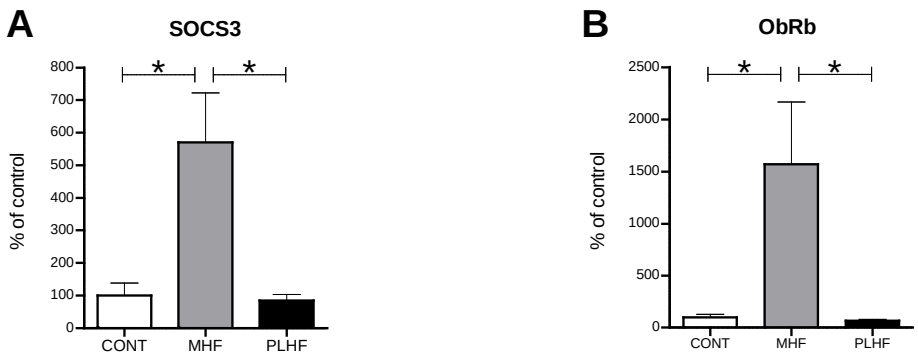


Figure 5.8: (A) Relative pancreatic expression of suppressor of cytokine signalling 3 (SOCS3) mRNA (n = 9-12) and (B) relative pancreatic expression of the leptin receptor long isoform (ObRb) mRNA (n = 5-10) in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100. * $P < 0.05$.

Although SOCS3 is a key component of the ObRb/JAK/STAT signalling pathway, no significant correlations were demonstrated between SOCS3 mRNA and any other gene in this pathway in PLHF offspring. In contrast, SOCS3 mRNA in MHF offspring exhibited correlations that were similar to those observed in Controls. The key observation in the SOCS3 correlation data was the striking difference between the MHF and PLHF groups (see Table 5.4).

Table 5. 4 Significant correlations of SOCS3 mRNA with mRNA levels of other genes

	CONT	MHF	PLHF
SOCS3 *vs* ObRb	$R^2 = 0.69$, $P = 0.08$	$R^2 = 0.87$, $P = 0.0008$	$R^2 = 0.03$, $P = 0.64$
SOCS3 *vs* STAT3	$R^2 = 0.68$, $P = 0.02$	$R^2 < 0.0001$, $P = 0.99$	$R^2 = 0.07$, $P = 0.39$
SOCS3 *vs* IR	$R^2 = 0.67$, $P = 0.02$	$R^2 = 0.02$, $P = 0.71$	$R^2 = 0.002$, $P = 0.90$
SOCS3 *vs* IRS1	$R^2 = 0.84$, $P = 0.01$	$R^2 = 0.58$, $P = 0.02$	$R^2 = 0.04$, $P = 0.51$
SOCS3 *vs* IRS2	$R^2 = 0.98$, $P = 0.002$	$R^2 = 0.58$, $P = 0.02$	$R^2 = 0.15$, $P = 0.21$
SOCS3 *vs* K^+ channel	$R^2 = 0.82$, $P = 0.03$	$R^2 = 0.93$, $P < 0.0001$	$R^2 = 0.002$, $P = 0.90$
SOCS3 *vs* Pdx1	$R^2 = 0.78$, $P = 0.009$	$R^2 = 0.91$, $P = 0.0003$	$R^2 = 0.07$, $P = 0.40$

All significant correlations in this table are positive relationships. (n = 5-13 per group)

In Control offspring, SOCS3 mRNA levels were positively correlated with six other genes that are key to the insulin and leptin signalling pathways (Table 5.4), although the relationship with ObRb did not reach statistical significance ($P = 0.08$). In MHF offspring, the correlation between SOCS3 and STAT3 in CONT animals was not present, but SOCS3 mRNA did correlate significantly with ObRb, IRS1, IRS2, K^+ channel, and Pdx1. In contrast, PLHF offspring did not demonstrate any significant correlation between SOCS3 mRNA and mRNA of those same 6 key genes in the insulin and leptin signalling pathways.

The relationship between SOCS3 and ObRb mRNA was not associated with any changes in leptin mRNA, the short form of the leptin receptor (ObRa) mRNA or STAT3 mRNA levels (Figure 5.9).

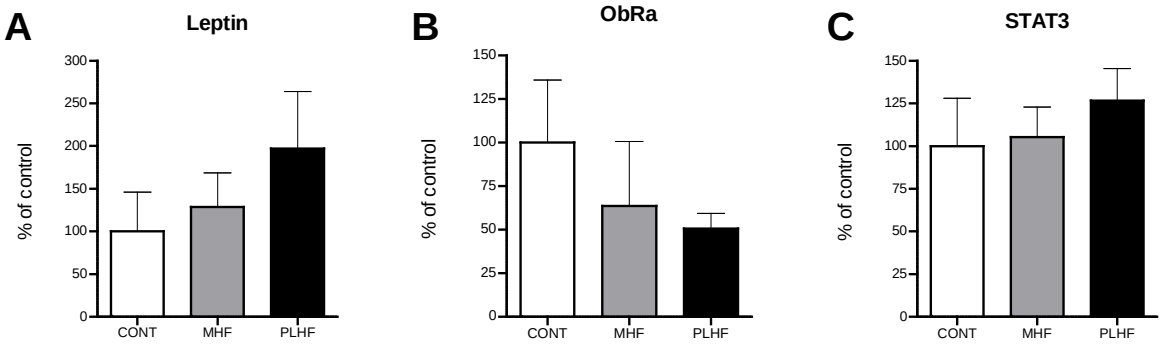


Figure 5.9: (A) Relative pancreatic expression of leptin mRNA (n = 6-12); (B) relative pancreatic expression of the leptin receptor short isoform (ObRa) mRNA (n = 6-10) and (C) relative pancreatic expression of signal transducer and activator of transcription 3 (STAT3) mRNA (n = 9-13) in the offspring of dams fed standard chow (CONT), a high fat diet from weaning and throughout pregnancy and lactation (maternal high fat, MHF), or a high fat diet during pregnancy and lactation only (pregnancy and lactation high fat, PLHF). Values are mean ± SEM and expressed as a percentage of control values, which have been standardised to 100.

STAT3 mRNA exhibited a positive relationship with SOCS3 in the CONT animals (see Table 5.4) and with Pdx1 mRNA in PLHF offspring (see Table 5.2).

Interestingly however, STAT3 mRNA levels correlated significantly and positively with IR mRNA, across all three groups of offspring. (STAT3 *vs* IR: CONT, $R^2 = 0.76$, $P = 0.005$; MHF, $R^2 = 0.56$, $P = 0.02$; PLHF, $R^2 = 0.55$, $P = 0.004$)

5.4 Discussion

The distinct patterns of mRNA expression resulting from our two maternal HF diet treatments indicate that different regulatory pathways underpin the leptin resistance common to both offspring groups. Despite the groups being phenotypically similar, mRNA expression patterns of key genes in the leptin receptor signalling pathways were distinct, dependent on maternal nutritional history. Maternal adiposity at the time of conception is a key determinant. This finding has important implications in the search for strategies to prevent the development of obesity and its associated epidemic of metabolic disease.

PLHF offspring The expected relationship between SOCS3 mRNA levels and other key genes, as exhibited by Control animals and largely maintained by the MHF cohort, was entirely absent in the PLHF group. This is striking, given how often SOCS3 inhibition of ObRb signalling has been proposed as a mechanism for leptin resistance, in a range of tissues (see Introduction) including pancreatic β-cells (208, 216). It is clear that leptin resistance in PLHF β-cells was mediated by mechanisms that did not involve SOCS3. In support of this, Peiser *et al.* (2000) found no change in SOCS3 expression in their rat model of HF feeding, although it is possible that the dietary regime they implemented was simply too low in fat to activate SOCS3 (427). Steinberg *et al.* (2004) reported a dissociation between insulin resistance and SOCS3 levels. In their DIO rat model, SOCS3 levels remained elevated despite leptin resistance having been reduced to normal through exercise (428).

However, PLHF offspring exhibited decreased PI3K mRNA levels compared to CONT animals (albeit, at just above significance levels). The ObRb/PI3K signalling pathway prolongs potassium channel opening, leading to decreased insulin exocytosis (see Figure 5.1), a direct action of leptin which inhibits insulin secretion (209). Moreover, the lowered PI3K mRNA levels in PLHF offspring were in direct relationship with a reduction in IRS1 and IRS2 mRNA, whose phosphorylation is coupled to PI3K activation. Of note, downstream of PI3K, PDE3B gene expression levels remained similar to Controls, suggesting that leptin resistance was achieved at the higher stages of this pathway. Thus, attenuation of the ObRb/PI3K signalling pathway may mediate leptin resistance in these hyperleptinemic PLHF offspring. Such a mechanism has been observed before, in hypothalamic neurons of DIO rats (429).

Both the IRS proteins and PI3K have other roles in the β-cell apart from the leptin signalling pathways. Both are also found downstream of the insulin receptor, in the IR/IRS/PI3K pathway which mediates the autocrine actions of the insulin-IR complex (430, 431). Our observed

attenuation of PI3K and IRS mRNAs did not, however, affect insulin synthesis – these animals were hyperinsulinemic and mRNA levels of their preproinsulins were elevated. Nor were mean mRNA levels of IR affected. (And since SOCS3 was not activated in these PLHF offspring, there was no SOCS3 mediated decrease in IR mRNA levels, as was seen in Controls.) Thus these mRNA changes of PI3K coupled to IRS likely reflect a source of leptin resistance.

Mean concentrations of ObRb mRNA in PLHF offspring were similar to Controls, but a significant inverse relationship existed between plasma leptin and ObRb mRNA levels in this group ($R^2 = 0.46$, $P = 0.03$), revealing a down regulation of ObRb numbers. A down regulation of receptor populations in the face of elevated ligand concentrations is not uncommon in resistant states. It is seen, for example, in the hypothalamus in the presence of hyperleptinemia (414-417, 432). There is a similar down regulation of hypothalamic ObRb in pregnancy, being a natural state of leptin resistance (361).

MHF offspring By contrast with the PLHF group, MHF offspring exhibited markedly elevated ObRb and SOCS3 mRNA levels *versus* Controls, and those mRNA levels were strongly correlated. Increased SOCS3 activity may be the mechanism of leptin resistance in these MHF offspring, via SOCS3 negative feedback inhibition of the JAK/STAT signalling pathway, as has been reported in the hypothalamus (420, 433). This inhibition of JAK/STAT is evident in that STAT3 mRNA levels plateaued against rising SOCS3 mRNA levels in MHF offspring, whereas in CONT offspring there was a positive relationship between SOCS3 and STAT3 mRNA levels.

Interestingly, mRNA levels of K^+ channel, and mRNA of IRS1 and IRS2 all rose in linear relationship with elevated ObRb mRNA, only in the MHF offspring. This supports the observation that, unlike the PLHF animals, attenuation of the ObRb-PI3K pathway was not the means of leptin resistance in the MHF group. Moreover, mean levels of PI3K and PDE3B mRNA within that signalling pathway in MHF animals were unchanged from Controls.

A rise in IRS1 and IRS2 mRNA associated with elevated ObRb and SOCS3 mRNA in MHF offspring may seem unexpected since SOCS3 is known to target insulin receptor signalling by interference with IRS action (434). However, IRS proteins are involved in both leptin signalling and insulin signalling, so altered gene expression levels may represent changes in either (or both) pathways, although IRS response to leptin is reportedly much lower than that seen with insulin (211). We saw a raised level of mean IRS1 expression in MHF offspring compared to Controls, plus there was a positive relationship between SOCS3 mRNA and both IRS1and IRS2 mRNA

levels in MHF offspring. This up-regulation of IRS1 expression may be related to the increased insulin production of the MHF offspring, since IRS1 helps regulate insulin secretion (435, 436).

SOCS3 is also reported to repress preproinsulin gene transcription (209), although other mechanisms clearly countered this action in MHF offspring, since mRNA concentrations of Ins1 and Ins2 remained elevated. This observation is confirmed by the significantly raised levels of circulating plasma insulin and the lack of any correlation in levels of mRNA between SOCS3 and Ins1 or Ins2.

Differences, MHF *versus* PLHF offspring There are other clear distinctions in mRNA profiles when PLHF and MHF offspring are compared. mRNA levels of the two IRS genes are elevated in MHF compared to PLHF offspring. This may reflect the attenuation of PI3K signalling as discussed above, where the IRSs are a direct link in that pathway. However, IRS2 also has a particular role in β-cell growth and development (437, 438). For example, activation of the important transcription factor Pdx1 is downstream of IRS2 (439-441), and both Pdx1 and IRS2 are significantly decreased in PLHF compared to MHF offspring.

Pdx1 transcribes a number of essential genes in the mature β-cell, including preproinsulin, glucokinase and GLUT2 (442) – and in our study the Pdx1 data exhibited a stark contrast between MHF and PLHF offspring groups. Mean levels of Pdx1 mRNA were significantly different between the offspring groups, with PLHF levels reduced compared to MHF. Moreover, the correlations between Pdx1 and other mRNAs occurred in quite separate clusters, MHF *versus* PLHF offspring. This suggests that Pdx1 activity and regulation were distinct between these two groups of offspring, and that this was determined by the difference in maternal nutrition.

In the MHF animals Pdx1 mRNA positively correlated with that cluster of mRNAs already described as strongly correlated together - ObRb, SOCS3, and IRS1&2 mRNA. In these offspring, Pdx1 appeared to be associated with the increased activity of the ObRb/SOCS3 signalling pathway which we hypothesise was the mechanism of leptin resistance in the MHF cohort. In the PLHF offspring, mRNA levels of Pdx1 were in correlation with IR and with Ins1&2 mRNAs. Pdx1 is known to have a regulatory relationship with insulin gene expression via the autocrine IR on the β-cell membrane (443, 444), and this correlation was present in the PLHF offspring, but interestingly, not at all in either the CONT or MHF groups.

Pdx1 is a transcription factor crucial for fetal β-cell development and maintenance in adulthood (445-447). Perturbed Pdx1 expression leads to impaired gene expression of insulin and GLUT2,

and increased β-cell apoptosis, to the point of loss of glycemic control, i.e. of T2DM (448, 449). Park *et al.* (2008) reported a progressive epigenetic suppression of Pdx1 transcription in a uterine artery ligation model of IUGR (107), evidence that expression of this gene can be modified by early nutritional environment. Our maternal HF diet did not produce differences in mean Pdx1 expression that were statistically different from Controls, but a significant difference emerged between MHF *versus* PLHF offspring. Moreover, Pdx1 mRNA correlated with distinct clusterings of gene transcription of mRNA in the two offspring groups. Interestingly, this difference in Pdx1 expression did not result in an outward phenotypic difference between the two offspring groups.

A decline in Pdx1 activity associated with obesity has been reported before, in the presence of glucotoxicity and lipotoxicity (450-453). The different patterns of Pdx1 activity revealed in our study may reflect similar phenomena, although the multiple correlations of mRNA within both offspring groups suggest that Pdx1 was still fully functional, and none of our animals had yet reached the stage of full-blown type 2 diabetes.

There was one gene only whose levels of mRNA correlated with Pdx1 in both MHF and PLHF offspring groups - and, remarkably, also with CONT - and that was the potassium channel, subunit Kir6.2. The K^+ channel regulates the membrane potential of the β-cell and is a crucial element in the insulin secretion mechanism (454). This may be the reason that Pdx1 mRNA correlated with the K^+ channel so strongly. Of note, Pdx1 mRNA tended to correlate with both plasma insulin and plasma leptin concentrations in CONT animals, whereas this relationship was not seen in MHF or PLHF offspring. Animals with defective K^+ channels are unable to regulate glucose effectively (455, 456). In a Zucker diabetic fatty rat model, hypothalamic K^+ channel gene expression was reduced in obesity and in diabetes (457). A similar result has been reported in the pancreas in the Zucker rat model (458), and in hyperinsulinemic mice (459). However, I believe we are the first to report an association between Pdx1 and K^+ channel mRNA levels. Interestingly, both genes are downstream of Foxa2 regulatory proteins (460).

Thus, MHF and PLHF offspring had different patterns of mRNA expression, suggesting that the leptin resistance that both exhibited was mediated via different signalling pathways downstream of the leptin receptor. However, the experimental difference between these two groups of offspring was <u>not</u> maternal nutrition during gestation and lactation – it was in the preconception diets of their mothers. Before breeding PLHF dams were raised on Chow, whereas MHF dams were raised on a HF diet. This resulted in MHF dams being significantly obese at the time of mating, and this degree of pregravid adiposity may be a key determinant of offspring mRNA

expression. Apart from the difference in maternal adiposity, offspring of both treatment groups in this gene expression study received identical *ad libitum* Chow diets throughout the study.

Maternal adiposity The difference in pre-conception diet between our treatment groups meant that the MHF dams were significantly heavier with increased adiposity than the PLHF dams at the point of mating and conception. Indeed, this increase in adiposity affected mating success – the fatter females had reduced oestrus cycling, as monitored by daily oestrus probing over 3 cycle periods (MHF dams 45% cycling *versus* PLHF dams 95% cycling), an observation that has been reported in rodent models before (135, 382). Lactation failure has also been reported in HF-fed animals (373, 461), although we did not see this in the current study.

One question that arises is: Was maternal adiposity itself the more powerful influence in the programming of offspring, or was maternal consumption of a high fat diet the primary mechanism leading to offspring obesity and leptin resistance? The two treatments produced different mRNA changes, suggestive of independent mechanisms, and these do not seem to be additive. Given that the offspring of MHF dams exhibited more mRNA changes, whereas mRNA profiles of PLHF offspring were little different from Controls, it could be argued that pre-conceptional obesity was the more perturbing influence on gene expression.

Other studies are broadly supportive of this. Patel & Srinivasen (2002) took newborn pups from Control pregnancies and made them obese by artificially feeding them a high carbohydrate diet during the suckling period (462). Though the animals received Chow after weaning, they remained obese and insulin resistant into adulthood. When females of this cohort were bred, they transmitted their programmed tendency to obesity and metabolic disease on to their offspring, even though their diet throughout gestation and lactation was standard Chow. This suggested that maternal obesity alone was sufficient to programme offspring, without any further exposure to HF nutrition (462). It has indeed been claimed that maternal obesity may be necessary for the programming effect of a HF diet on offspring. White *et al.* (2009) compared offspring of obese HF-fed dams, with those from dams who were pair-fed measured amounts of HF diet iso-caloric with the Chow-fed Control dams. Offspring of the obese dams exhibited the expected metabolic programming, but the offspring of the HF pair-fed dams were no different from Controls (463). The authors concluded that maternal consumption of HF alone, in the absence of obesity, was insufficient to induce programming of obesity in offspring. Unlike White *et al.* we did see programming from a maternal HF-diet alone – our PLHF dams were not obese at mating, nor were their body weight changes through pregnancy and lactation any different from Controls. This was likely due to our provision of *ad libitum* HF diet, as opposed to White *et al.*'s restricted

pair-feeding. Nevertheless, their study lends support to our claim that there are different mechanisms underlying the similar programming outcomes of our MHF *versus* PLHF treatments, and moreover, that the maternal obesity of the MHF dams produced a more perturbing influence on gene expression.

Investigations into obesity in human mothers are also not inconsistent with our findings. A recent study examining the perinatal risk factors related to childhood obesity found that maternal pregravid BMI was the strongest predictor of obesity in children assessed at age 9 years, and this influence was independent of birth weight or maternal glucose status (464). It may be that obese women bring a pre-existing low-grade chronic inflammatory state to conception, which may then perturb the usual adjustments in insulin and lipid metabolism of pregnancy, and lead to increased fetal fat deposition (465, 466). Indeed, in rats it has been shown that nutritional disturbance applied solely during the pre-implantation stage (before day 4.5 post mating) produced lasting programming changes in body weight and blood pressure (308). Pregravid obesity is thus a distinct risk factor for offspring obesity and metabolic disease. It is also a potentially preventable factor.

Conclusions This study has demonstrated the enduring deleterious effects of a maternal HF diet during early windows of development. Maternal HF feeding both before conception and/or through pregnancy and lactation induced leptin resistance in adult offspring, irrespective of postnatal diet. However, although both models of maternal HF feeding programmed obesity in offspring and pancreatic resistance to elevated plasma leptin, our data highlight distinct mechanisms for leptin resistance between the two cohorts, mediated by different pathways downstream of the leptin receptor. These data reinforce the importance of maternal nutrition both before conception and during pregnancy and lactation, and demonstrate that maternal obesity at the time of conception can permanently alter gene expression in important metabolic pathways, thus adjusting offspring phenotype and response to post-weaning nutritional environments. It is recommended that pregravid weight control become a key factor in strategies seeking to prevent the programming and transgenerational transmission of obesity.

Chapter 6 - Discussion

The science of developmental programming is based on the observations that environmental changes can reset the developmental path during early life leading to metabolic, cardiovascular, and behavioural disorders in later life. The pathogenesis is not based on genetic defects but on altered genetic expression as a consequence of an adaptation to environmental changes during early life development. Developmental programming research has thus offered a novel approach to investigate the mechanistic basis of common metabolic disorders such as obesity and type 2 diabetes, which in human populations predominantly arises from environmental factors.

The results incorporated in this thesis, using nutrition models at both ends of the "U" shaped nutrition curve, have reinforced the human epidemiological evidence for developmental programming of adult-onset diseases and, furthermore, have shown that exposures to altered nutritional planes during different critical windows of developmental plasticity elicit different metabolic outcomes. The current work has demonstrated that altered maternal nutrition during critical windows affects phenotype, gene expression and metabolic profile of offspring and provides further empirical evidence to support the DOHaD hypothesis.

Developmental plasticity is typically a phenomenon occurring in the early period of an organism's life during periods of rapid cell division. Factors that impact the organism during these window/s of plasticity, whether for good or for bad, can have permanent and irreversible effects, for the lifetime of the organism. Early events can exert enormous influence because they can become fixed and may be amplified in adult life. Indeed, the DOHaD hypothesis asserts that the effect of the developmental programming may not become truly obvious or symptomatic until adulthood: T2DM is typically an adult-onset condition. Before the DOHaD concept, that is, until the connection between early life events and subsequent adult health was observed, the metabolic syndrome was attributed primarily to adult lifestyle factors or to genetic background.

6.1 The 'thrifty phenotype' hypothesis

The first attempt to construct a mechanistic framework to explain the epidemiological associations between early development and later disease was the 'thrifty phenotype hypothesis' coined by Hales and Barker in 1992 (467). The thrifty phenotype hypothesis grew out of observations that small birth weight individuals had increased risk of developing decreased insulin sensitivity and T2DM in adult life. Poor fetal nutrition, due to maternal malnutrition or

placental insufficiency, activated a form of fetal proportioning and selective distribution of available nutritional resources, which protected the development of the brain at the expense of other organs, including the pancreas. This was an adaptive response, to ensure fetal survival and reproduction in an adverse environment – it enhanced energy efficiency and storage, and advanced reproductive maturation. The apparently perturbed development of the pancreas was key to the proposed mechanism - reduced capacity for insulin secretion, plus insulin resistance in peripheral tissues as a way of preserving blood glucose for the brain. However, when this thrifty phenotype encountered the challenge of aging or of obesity the end result was T2DM and/or other features of the metabolic syndrome (18).

The thrifty phenotype is an adaptive response. It makes sense in a harsh environment to be born with a small body, with reduced activity level and reduced metabolic rate. These characteristics are not necessarily pathological; since they all save energy in an environment of deprivation, they may be regarded as appropriate and adaptive in that setting (468, 469). The thrifty phenotype is therefore a normal adaptive response, a response contained within the wider repertoire of developmental plasticity, a response cued by less-than-abundant maternal nutrition.

6.2 Predictive adaptive responses

Originally the hypothesis of the thrifty phenotype applied solely to settings of IUGR, mainly due to maternal undernutrition, such as the Dutch Hunger Winter. It was harder to apply it to other maternal nutritional insults such as HF feeding. Thus, it has been felt that the responses of the fetus needed to be viewed in a broader context (470), perhaps by utilising some of the concepts of evolutionary medicine (471). Typically, adaptive responses were seen to have immediate benefits; they ensure the immediate survival of the fetus, and these benefits may be limited to the immediate *in utero* environment. Gluckman and Hanson, however, proposed another subset of adaptive responses (78, 79). These were adaptations occurring during *in utero* life that did not necessarily confer an immediate advantage, but which would benefit the fetus in the future - typically in postnatal life, beyond the womb. They termed these ‘predictive adaptive responses’ or PARs (283, 472). The word ‘predictive’ was important – these adaptive responses had a purpose projected into the future, to convey an advantage in an uncertain but anticipated postnatal environment. Moreover, this function, this facility to make anticipatory adaptations had been naturally selected across successive generations because it had aided survival and reproductive success to species inhabiting a world of varying nutritional environments and naturally subject to uncertainty. PARs operate within a single generation making possible a very rapid adaptation to any immediate change in nutritional environment. They are as equally rapidly reversible, within a

generation or two, should environmental conditions revert or further change. The thrifty phenotype is in fact an example of a PAR.

The value of a PAR depends on the accuracy of its prediction. If the prediction is on target, and the developmental adaptation is aligned with the future nutritional environment, then survival and breeding are optimised in those conditions. This favourable alignment is termed ‘match’. Clearly, match is most effective in a world that is relatively stable across generations, so that prenatal cuing consistently signals postnatal conditions. However, inappropriate interpretations of prenatal cues or changes from that immediate environment may result in a ‘mismatch’ between prenatal predictions and postnatal reality. As a result, the predictive adaptive responses may ultimately prove disadvantageous in later life, leading to an increased propensity for chronic non-communicable disease in adulthood and/or the inheritance of risk factors (283, 473). It is the magnitude of mismatch that determines risk of disease, rather than the absolute level of either developmental or postnatal nutrition. The classic example of mismatch is when a ‘thrifty’ fetus, programmed to conserve energy and with physiology differentially tuned accordingly, enters into a postnatal environment of nutritional abundance for which it is ill-prepared. In rodents and in humans this situation leads to obesity, insulin resistance, T2DM and other symptoms of the metabolic syndrome. Hales and Barker believed that such environmental factors were “the dominant cause” of T2DM.

It is proposed that intrauterine nutrition supply is an important predictive cue (43), and therefore, in the current set of studies, we manipulated maternal nutrition through different developmental periods in order to investigate and to compare the effects on glucose/insulin metabolism emerging in the offspring in later life.

The PAR hypothesis is not without its critics (474, 475). For example, it is not possible at this stage to determine whether some observed elements of developmental programming are either adaptive or predictive. For example, when the thrifty phenotype is born with reduced pancreatic or renal functional capacity – is that truly adaptive, or is it simply a deficit? It is also difficult to determine exact cues or ‘sensitive windows’ with any accuracy. Some PAR mechanisms are thought to be operative even before the implantation of the embryo in the uterine wall (308, 309, 476). In humans, there is a long time interval between pre-implantation and reproductive maturity, attenuating the value of a predictive adaption. Jonathon Wells has argued against the word ‘predictive’. He suggests that maternal cuing of the fetus is not so much about the current nutritional environment, which may only reflect short-term fluctuations. Instead cuing reflects maternal phenotype which in turn depends on her matrilineal history (the transgenerational effect,

whereby the maternal physiology is shaped by her mother's and her grandmother's programming). Thus, instead of looking forward in prediction, the fetus looks backward, in the assumption that conditions will remain relatively similar to those of recent ancestors. Wells maintains that maternal history is likely to be a more accurate signal for development than immediate environmental conditions, which are usually buffered from the fetus by maternal fat stores (477, 478).

Although the PAR hypothesis remains yet to be fully accepted, all agree on key aspects of the DOHaD phenomenon – that maternal cuing and shaping of offspring physiology occurs during development and that this will affect the organism throughout the rest of its life. That a mismatch between early development and later environment will represent a challenge to health and well-being, is also held in common. The modern energy-dense diet is seen by both sides of the argument as a nutritional environment to which humans may not be ideally historically suited. All proponents declare the value of optimising nutrition for both mother and offspring.

The current study did not provide us with the opportunity to examine matrilineal experience, but it did allow us to ponder the implications of the PAR hypothesis. Several of our treatment groups are examples of either match or mismatch between early developmental *versus* postnatal nutritional environments.

6.3 Developmental "match" or "mismatch"

The studies presented in this thesis utilise rodent models of altered maternal nutrition at both ends of the "U"-shaped nutrition curve; moderate maternal undernutrition and a maternal high fat diet. Further, with each nutrition model, different windows of maternal nutritional challenges are utilised coupled with two planes of post-weaning nutrition. These balanced experimental paradigms allowed the investigation of "critical" windows on phenotype development and sex-specific effects therein.

Maternal undernutrition Prevention of catch-up growth within our undernutrition model preserved metabolic health into adulthood. UNPL animals, with matched 50%UN nutrition through both pregnancy and lactation were not more obese that Controls and exhibited a leptin sensitivity better or no different from Controls.

UNPL offspring were born small, were the lightest of all groups at weaning, and they remained small throughout life. Although the addition of a post-weaning HF diet permitted extra weight

gain, this increase was solely in adipose tissue. The weight of lean tissue mass in HF-fed adults remained the same as those fed Chow, in both males and females.

In body composition, UNPL offspring had similar %body fat to CONT animals, irrespective of post-weaning diet or gender. Despite this overall similarity, UNPL male and female offspring had lighter retroperitoneal fat pads relative to total body weight than CONT animals. This suggests that body fat may be differently distributed in UNPL *versus* CONT animals. In humans who had experienced *in utero* undernutrition such as victims of the Dutch Hunger Winter, there was a tendency to greater adiposity being deposited centrally – as visceral or omental fat. However, this may not apply to our UNPL cohort, since the Dutch Hunger Winter was not an example of 'matched' nutrition. The longer duration of the WWII Siege of Leningrad may provide a better example of matched undernutrition in humans, and among those subjects studied no association between intrauterine starvation and metabolic syndrome was found. This was thought due to the unavailability of food to provide for catch-up growth (479). Koupil *et al.* (2007) also investigated victims of the Leningrad Siege - they noted a tendency to adult hypertension and ischemic heart disease in subjects exposed to the siege as children or adolescents, but these were not *in utero* exposures to the famine, and the effects were attributed by the authors to 'severe stress and starvation' (480).

UNPL offspring had similar leptin levels to CONT animals, in Chow-fed groups, both males and females. In the HF-fed cohorts, male UNPL offspring had significantly lower leptin levels than CONT, with females no different from Controls. Thus leptin sensitivity, as measured by insulin:leptin ratio, was equivalent to or raised over comparative Controls. CONT male offspring showed a significant post-weaning diet effect in their insulin:leptin ratio. Interestingly, only the UNPL males mirrored this - further evidence of the similarities between the CONT and UNPL groups, perhaps because both experienced matched pre- and postnatal nutritional environments.

Overall, the UNPL phenotype lends support to the PAR hypothesis that matched pre- and post natal nutritional planes result in a healthy animal with less difference to Controls – despite the lifelong reduction in growth and size seen in the doubly undernourished cohort. In a slightly different context, if we had compared the UNPL animals with the UNP offspring (instead of against CONT), this conclusion would have been framed as 'prevention of catch-up growth' which ameliorated the extremes of the UNP phenotype.

Effects of a maternal obesogenic environment The PLHF pups received a high fat nutritional environment both pre- and postnatally, and thus are an example of nutritional "match"

in an obesogenic environment. Although the MHF dams produced offspring of a similar phenotype, they are not a clear-cut example of nutritional "match" because of the added factor of their pre-conceptional adiposity, so the following discussion focuses on the PLHF offspring.

Despite a slightly longer gestation periods PLHF pups were born smaller that CONT. They exhibited accelerated growth through lactation, and by weaning outweighed Controls. Post-weaning weight gain continued significantly increased, irrespective of postnatal diet. Thus, the PLHF offspring had a very different growth trajectory compared to Controls.

PLHF offspring had significantly increased percentage body fat compared to CONT, but only in the Chow-fed offspring. It may be that those PLHF animals which continued on the HF diet post-weaning were more tolerant of its obesity-inducing effects. This could suggest evidence for 'match'. The case for this was stronger in PLHF males, where diet-induced obesity was significantly less than CONT. However, this narrowing of diet-induced obesity was simply due to their propensity to gain weight, their elevated weight gain on standard Chow. An alternative explanation might be that all the HF-fed males were reaching an adiposity "threshold". While this alternative sounds plausible, given the 50-60+% adiposity of some of these animals, there still remained a > 10% difference in body fat between CONT-HF *versus* PLHF-HF males, with no apparent adiposity threshold attained.

The maternal HF diet altered metabolic hormone levels in offspring: plasma insulin was elevated in males and females. Plasma leptin levels were significantly raised in both sexes, and further increased by a post-weaning HF diet. However, insulin:leptin ratios were not different from CONT in either males or females. This raises two questions: Were these animals coping with a HF post-weaning nutritional environment better than other animals? Was this due to developmental programming from early HF exposure?

In answer to the first question, the PLHF animals display some of the hallmarks of the metabolic syndrome. Males and female adult offspring had raised insulin levels – indicative of insulin resistance. Leptin levels were elevated, in line with increased adiposity. Moreover, elevated leptin plus elevated insulin levels are suggestive of leptin resistance in β-cells and perturbed adipoinsular axis activity. Remarkably, however, the relationship between plasma insulin and leptin remained linear throughout: despite circulating levels of both hormones being dramatically elevated the tight correlation between them held, and Insulin:Leptin Ratios stayed similar to Controls. Thus it cannot be asserted that the adipoinsular axis had become 'perturbed' or dysfunctional; in fact the relationship between insulin and leptin is revealed as robust, even if

raised levels are indicative of tissue resistance and may signify the beginnings of metabolic disease.

To answer the second question: Maternal HF feeding in the PLHF dams led to reduced birthweight in pups despite increased gestational length. This suggests a maternal-fetal supply problem during gestation. Moreover, the pups' growth trajectory, from IUGR to catch-up growth, resembles the pattern of the thrifty phenotype, which typically leads to the metabolic syndrome in adulthood. Although high fat feeding in gestation is not the same as undernutrition, both are examples of malnutrition, and both can lead to obesity and the metabolic syndrome. Whether or not the same biological mechanism underlies these similar outcomes remains uncertain.

Thus from our data I conclude that maternal HF feeding is unlikely to induce a predictive adaptive response, and should rather be viewed as a nutritional insult. Gluckman and Hanson argue that a PAR may extend its influence across several generations (481). To test this, an F2 generation would need to be raised, although breeding the F1 generation might have proved problematic, given the estrus cycle irregularities and poor reproductive success rate of the obese MHF dams. A poor reproductive rate is not a good signifier of a positive PAR, and a true nutritional insult is unlikely to diminish across generations.

Nutritional Mismatch The UNP and UNL offspring represent different windows of mismatch, allowing us to compare mismatched nutrition through pregnancy *versus* lactation.

UNP pups were born small, exhibited rapid catch-up growth and developed excessive adiposity as adults, with raised leptin levels, although plasma insulin remained no different from CONT. UNP offspring also showed more overall changes in relative organ weights than other groups. This phenotype was not unexpected: it is the classic mismatch model that has been shown before (296, 324), and a classic example of the thrifty phenotype. That we have reproduced it so successfully with our maternal treatment of 50% undernutrition is a validation of the animal modelling in this current study, which inspires confidence in our overall results.

By contrast, the UNL offspring had normal birth weights, but they weighed significantly less than Controls at weaning. They experienced an increased energy intake for the first week on an *ad libitum* post-weaning diet, and UNL growth curves eventually came to be no different from CONT growth curves, in both male and female Chow-fed animals. However, there were gender-specific differences in adult UNL offspring. UNL males had similar percentage body fat and fasting plasma insulin and leptin concentrations to Controls. In contrast, in UNL females there

was a significant reduction in percent fat mass compared to the CONT group, with no changes in plasma insulin or leptin compared to CONT. Moreover, the insulin:leptin ratio was elevated in male UNL offspring *versus* Controls, but not in females. This suggests that prevention of catch-up growth in females can confer lasting beneficial effects on body composition. An examination of the Fat:Lean ratios (g) supports this: the two groups where catch-up growth was prevented exhibited ratios that were similar to Controls, whereas the UNP group had Fat:Lean ratios significantly higher than both those groups, in both males and females.

Since similar findings were seen in both UNL and UNPL offspring, the benefits of preventing catch-up growth are independent of the level of maternal nutrition during pregnancy. This indicates that: The tendency to 'thrifty' adipose deposition was formed *in utero*, was present in the neonate, and active during lactation. During the window of lactation this early programming remained plastic, that is, open to modification, so that prevention of catch-up growth at this stage could circumvent the extremes of the UNP phenotype. Where catch-up growth during lactation was prevented, the phenotype in adulthood was similar (or for some parameters, improved) than Controls.

This developmental window appeared to close at the end of lactation: even with access to an unrestricted Chow diet after weaning, these animals remained phenotypically similar to Controls. Thus, there was a period of metabolic plasticity during lactation which closed at weaning. Diet before weaning (particularly in relation to gestational nutrition) affected adult phenotype in a way that post-weaning diet did not. Other studies confirm these observations. Desai *et al*. (2007) conducted a mismatch study, gestation *versus* lactation, with a timeframe for undernutrition from day 10 of pregnancy only, and found that timing of catch-up growth determined programming of phenotype (337).

Different windows of nutritional challenges and different phenotypic outcomes When UNP offspring are compared with UNL offspring - these being the two groups that experienced mismatched nutrition - it is evident that undernutrition during pregnancy followed by catch-up growth induced a significantly increased adiposity and a depressed insulin:leptin ratio, indicative of leptin resistance. On these grounds the UNLs were the healthier cohort.

An examination of raw unadjusted weight of lean tissue, as delivered by the DEXA data, was revealing, as follows. In males, unadjusted weight of lean tissue demonstrated an overall maternal diet effect of CONT > UNP and UNPL groups), but interestingly, <u>not</u> CONT *vs* UNL. This suggests that it was undernutrition during gestation that affected development of lean tissue, as

has been reported before (482-484). The UNL group, however, maintained a similar lean tissue mass to Controls. The slight weight advantage that the CONT animals had over the UNL groups through to the end of the study was due to increased fat, not lean tissue mass. This is further evidence that undernutrition during lactation alone had less harmful effects than undernutrition during pregnancy alone.

It is also obvious that males in general had a heavier unadjusted lean mass (since male rats were bigger than females). However, when gender effects were examined in the %lean data, the difference between males *versus* females was only significant within the CONT and UNL groups, but not within the UNP or UNPL animals. This observation again suggests that the UNL condition, of all the undernutrition treatment groups, was the one most similar to Controls.

In conclusion, undernutrition during pregnancy followed by the mismatch of abundant nutrition resulting in catch-up growth had the most severe effects on adult health and susceptibility to disease. Prevention of catch-up growth, as seen in both UNPL and UNL cohorts, protected against development of metabolic syndrome symptoms, so that these offspring were either no different from Controls, or had improved metabolic profiles. It is perhaps not surprising that malnutrition during pregnancy alone had greater effects on subsequent adult health than malnutrition during lactation, since it is a generally held principle in developmental plasticity that the earlier the perturbation or adaptation, the greater the developmental effect or amplification.

6.4 Is catch-up growth detrimental?

It appears that the programming of adult obesity and the metabolic syndrome requires not only an *in utero* nutritional insult, but also a necessary component of postnatal catch-up growth. This is clearly seen in comparing the various cohorts in the undernutrition branch of our study. The UNP offspring, born IUGR followed by abundant postnatal nutrition, leading to increased adiposity in adulthood, replicated one of the most consistent findings in developmental programming studies. By contrast, the IUGR offspring in the PLHF group, where catch-up growth was prevented, were no different from Control animals in terms of adiposity, or plasma insulin and leptin levels – indeed, female adults exhibited an increased leptin sensitivity[6] compared to CONT. A similar result was seen in our UNL group, where again catch-up growth was prevented. Here too there was a gender effect: although both sexes had similar body weights to Controls, only the females had reduced proportional fat mass.

[6] Note: Leptin responses were not directly tested; thus leptin sensitivity has been inferred from plasma hormone levels and adiposity indices.

The importance of catch-up growth in programming later metabolic health has also been documented in humans. Those most at risk of the metabolic syndrome were those born small who then experienced rapid post-natal growth (485, 486). In the 1934-44 Helsinki cohort, the development of T2DM was associated with lower birth weight plus rapid growth in weight and height through childhood (8). Breast-feeding has been especially recommended for low birth weight babies, specifically because it restrains infant weight gain (compared to formula feeding) and thereby prevents excessive adiposity developing in later childhood (487, 488).

It seems, however, that it is usual for low birthweight babies to exhibit rapid weight gain during the first year of life, so that the majority will obtain a BMI within the normal range of childhood well within their first year (489-491). This may in fact represent a physiological and homeostatic norm, an attempt to restore an intrinsic genetic potential for body size and weight, which had been held back by poor intrauterine conditions. This parallels the normal rapid increase in BMI seen in babies of normal birthweight, an increase that persists only until about one year of age, but is then followed by a gradually falling BMI until about 6 years of age, when the phenomenon of adiposity rebound occurs (492). Adiposity rebound is a normal increase in fat mass and BMI; it is not a 'physiological' catch-up of growth. However, when adiposity rebound occurs earlier than 5 years of age, there is increased risk of obesity in adolescence and in adulthood (9, 493). It may be then, that early catch-up growth is a normal adaptive phenomenon that only becomes problematic when it is prolonged or delayed past one year, in which case it resembles early adiposity rebound.

Thus it may be the timing of catch-up growth that is critical for later adverse consequences. For example, Barker *et al* (2005) showed that heart disease in adulthood was associated not just with low birth weight *per se*, but with thinness at age two followed by accelerated weight gain – ie, a model of perturbed catch-up growth (494). Moreover, Barker *et al.* found that low birthweight babies whose catch-up growth occurred within their first year were <u>not</u> at greater risk of coronary disease in adult life (494). Similarly, in a study by Ibanez *et al* (2008) looking at post-catch-up growth, SGA and AGA infants were not different in terms of body weight or adiposity at age two, but SGA infants went on to increased BMI, central adiposity and insulin resistance by age six (495). The regulation of the timing of catch-up growth and of the onset of adiposity rebound are unknown, and how it is connected to the intrauterine events that led to low birthweight is also yet to be determined. Nevertheless, there is an association between thinness at birth plus earlier adiposity rebound leading to increased adult adiposity. It is as if the process of prenatal growth

restriction has an influence on the postnatal growth trajectory, that the two are linked and must be taken together in understanding developmental programming.

The notion that catch-up growth may have adverse consequences is embedded in the 'thrifty gene' and 'thrifty phenotype' hypotheses as described earlier. Neel (1962, 1999) proposed that there was an evolutionary advantage in having a genome adapted to efficient energy storage. In times of abundant food supply surplus nutrients could be stored as fat which would sustain the organism during times of scarcity or famine. These efficient 'thrifty' genes were selected in early hominid populations where food supply fluctuated, but are no longer an advantage in modern populations where food supply is constant or abundant. (Neel's argument is not without its challengers – on the basis, for example, that still-surviving hunter-gatherer cultures do not exhibit fat acquisition during times of seasonal plenty (496). Moreover, such a pattern of seasonally fluctuating BMI would be inconsistent with the evidence for a defended set-point for body weight (388).) Neel's insight is that the human genome may carry historical shaping that works against health in some individuals in modern nutritionally abundant or nutritionally transitioning societies. Stoger (2008) argues that 'metabolic thrift' is so fundamental to survival and fitness that it is encoded in all genomes, both human and those much earlier (497). If all organisms encode metabolic thrift, if all human genotypes are equally thrifty, this would account for the lack of success in the search for the gene variations conferring thrift (498).

The thrifty phenotype hypothesis (467) proposed that the factors leading toward obesity and T2DM in adult life were developmental and environmental (e.g. nutritional) rather than predominantly genetic. If all human genotypes are equally encoded for 'metabolic thrift' then environmental and nutritional events may interact adaptively or perhaps mal-adaptively with that trait. An even more recent refinement is the 'thrifty epigenotype' hypothesis which proposes that environmental factors shape gene expression via epigenetic mechanisms, which persist into adult life (497).

Evidence for the thrifty hypothesis was found in fetal insulin levels. IUGR rats exhibited lowered plasma insulin levels and increased insulin sensitivity at birth (115, 157). It was suggested that reduced fetal energy substrate levels induced raised insulin sensitivity to promote growth and efficient utilisation of nutrition, and this was a thrifty mechanism and appropriate for the *in utero* environment of restricted nutrition. This elevated sensitivity was then responsible for the increased weight gain evident as catch-up growth, occurring when postnatal nutrition levels were greater than those *in utero*.

Fetal growth restriction in and of itself may produce raised insulin sensitivity, regardless of actual birth weight (491). Moreover, growth restricted newborns have less fat, so their leptin levels tend to be lowered. We observed both lowered insulin and leptin levels in low birthweight neonates, the P2 pups born to dams who received HF through pregnancy and lactation. These changes are proposed to predispose neonates to increased weight gain if the postnatal nutritional environment was favourable.

It is a striking observation that the pups born to the two maternal high fat treatment groups (that is, the MHF and PLHF offspring) showed broad similarities to the UNP pups – that cohort whose mothers were fed only 50% of Control chow levels during gestation. Both MHF and PLHF dams, and UNP dams produced pups with birth weights significantly lighter than Controls. Both sets of offspring exhibited catch-up growth during lactation, in that UNP offspring had almost reached the same body weight as Controls by weaning, while the MHF and PLHF pups were already significantly heavier than CONT by weaning. At the end of the study period, both UNP offspring and the MHF and PLHF offspring had significantly more adiposity than Control offspring. It appears that the very different maternal nutritional treatments during early development – 50% undernutrition *versus* high fat feeding - had produced a similar outcome in their respective offspring, and had generated similar growth trajectories resulting in similar end-phenotypes.

It may be therefore, that common mechanisms underlie this phenomenon. It may be that sub-optimal maternal nutrition, irrespective of whether it is undernutrition or exposure to high fat, can lead to intrauterine growth retardation (IUGR) in pups. IUGR coupled with unrestrained availability of postnatal nutrition resulted in catch-up growth, which set in train a trajectory that led to adiposity in adult life.

There were differences between the UNP *versus* MHF and PLHF offspring: the MHF and PLHF offspring were significantly hyperleptinemic and hyperinsulinemic compared to Controls, in both males and females, whereas the UNP offspring had elevated plasma leptin compared to Controls in females, but not in males, and plasma insulin was similar to Controls in both males and females. The MHF and PLHF offspring were significantly heavier than their UNP counterparts at weaning and PLHF offspring were significantly more obese than UNP at the end of the study. These differences in adiposity likely accounted for the different leptin levels, which may in turn have influenced plasma insulin levels, since these two hormones demonstrated a robust correlation with each other.

(Note: the MHF and PLHF offspring have been grouped together throughout this discussion, taken as one greater grouping rather than as two individual groups. This is because no real differences existed between them in broad adult phenotype, as described in Chapter 4. However, despite this phenotypic similarity, we have shown that the genes underpinning key elements of insulin and leptin signalling in pancreatic cells had very different patterns of mRNA expression between MHF *versus* PLHF offspring. Yet although there were significant mRNA differences, the phenomenon of intrauterine growth restriction plus catch-up growth appears to have dictated the broad phenotypic outcome.)

If there is a common growth trajectory for all these offspring groups, despite the very different nutritional experiences of their mothers, what would be the underlying mechanism? There appears to be a common developmental experience across these different offspring groups, namely intrauterine growth restriction plus catch-up growth: is there a common underlying mechanism driving this shared developmental experience leading to the same end result of elevated adult adiposity? Or is it simply coincidental that different maternal nutritional backgrounds produced similar offspring outcomes?

Moreover, it is clear that what underpinned the increased adult adiposity was not excessive food consumption. Energy intake in all these animals (kcals/gram body weight) was no different from Controls, in either UNP or MHF and PLHF offspring, irrespective of sex or post-weaning diet. If energy intake remained similar, yet adipose tissue stores expanded, it follows that there must have been a shift in metabolic regulation, in energy utilisation and in fat deposition. Any proposed mechanism seeking to explain the commonalities of these different offspring groups must account for this shift in metabolic physiology, this resetting of the regulators of fat deposition.

6.5 Mechanisms

Various mechanistic explanations have been offered for this phenomenon, and these will be briefly examined next. No one mechanism has yet emerged as definitive of programming, and many of these proposed mechanisms may be applicable to this current study.

It has been proposed that IUGR causes a selective distribution of available resources, favouring the development of some organs over others (86). In particular the brain was preferred above organs that regulate metabolism, such as the pancreas, the liver and skeletal muscle: this has been termed ‘brain sparing’ (86). Changes that occurred in the neonatal rodent pancreas as a result of a

maternal low protein diet included reduced islet size and β–cell mass, with reduced pancreatic insulin content (89, 499). Maternal 50% undernutrition had a similar effect (157). Such impairment of pancreatic development and function means that there is less functional reserve to meet the challenges of metabolic or oxidative stress that may occur in later adulthood, with aging or with overweight. Interestingly, a maternal diet rich in fat had a similar effect on fetal pancreatic development, resulting in reduced β-cell volume and numbers, and hyperglycemia in neonates (374, 500). A similar study by Siemelink *et al.* (2002) found that a maternal saturated fat diet reduced islet numbers in 12 week old offspring, with an exaggerated insulin response to glucose load (501). This mechanistic model, relating early impairment of organ development to adult disease, has also been proposed to underlie other elements of the metabolic syndrome – for example, reduced nephron numbers due to IUGR may contribute to subsequent hypertension (85, 502), and undernutrition may reduce skeletal muscle development leading to permanently lower lean tissue mass in adulthood (503, 504) which may influence insulin disposal.

It is thus possible that impaired pancreatic organogenesis may contribute to the common phenotype observed in our study, which produced IUGR pups from very different maternal dietary treatments. We have not yet examined islet size or quantified β-cell mass or morphology in our animals, and this is a limitation of the study thus far, although this work is currently underway.

It was the wider purpose of the current study to validate a model of developmental programming and then to think mechanistically, starting with mRNA levels in key signalling pathways in the obese offspring of the dams in the maternal HF treatment groups. Although we have demonstrated striking changes in the mRNA of the insulin and leptin signalling pathways in pancreatic tissue, we also find ourselves in a teleological position: the fine detail of the mechanisms in early development that might account for these epigenetic changes remains to be elucidated.

Another proposed mechanism involves the hypothalamic-pituitary-adrenal (HPA) axis and the influence of glucocorticoids on development (46). Glucocorticoids are essential for tissue and organ maturation (505) and maternal milk production (506). Exposure to high levels of maternal glucocorticoids *in utero* led to high blood pressure in rat offspring (83, 507), and injections of synthetic glucocorticoids to pregnant rats reduced birthweight of pups (508, 509) and led to fasting hyperglycemia in adult offspring (510). Maternal undernutrition in rodents has been shown to programme a chronically hyperactive HPA axis in adult offspring with high circulating glucocorticoid levels, associated with behavioural changes (511, 512). Furthermore, utilising

adrenal tissues from the current study, changes were detected in adult offspring in mRNA levels of key enzymes that regulate adrenal steroidogenesis, and this was dependent on maternal dietary background (513). Thus, it seems probable that early changes to the HPA axis also have a role in the developmental programming phenomenon. However, how HPA axis changes might contribution to the formation of a 'thrifty phenotype' is as yet not fully defined.

What are the roles of insulin and leptin in developmental programming? Plasma leptin has an important function in facilitating hypothalamic neuronal growth in those brain areas that regulate appetite and satiety (277, 335). NYP/AgRP projections between the arcuate (ARH) and the paraventricular nuclei (PVN) of the hypothalamus are not fully formed until P15-16 in rats and the growth of these projections is under the control of leptin, the so-called neonatal leptin surge (514). For example, in ob/ob mice which lack the gene to synthesise leptin these hypothalamic projections become disordered, but administration of exogenous leptin can rescue the pathways, if given during the critical neonatal period. It is ineffective if given in adulthood (277). This critical window of brain development is therefore vulnerable to nutritional changes that perturb leptin levels, and this will have enduring effects on the CNS circuitry that regulates food intake and body weight.

IUGR has been shown to alter the timing of the leptin surge in a 70% undernutrition mouse model, and early postnatal catch-up growth led to a premature leptin surge (111). When control mice were administered exogenous leptin in doses that mimicked the premature leptin surge of the IUGR animals, they developed the same phenotype as the IUGR mice, that is, adult obesity and leptin resistance (111). It has also been shown that leptin administered to IUGR pups of undernourished dams can rescue the usual obese phenotype, so that adult offspring were no different from Controls (123). Moreover, offspring of obese rats also had an altered neonatal leptin surge: these rats showed an amplified and prolonged surge compared to Controls, with obesity in adulthood and permanent central leptin resistance (515). These studies are clear evidence of the importance of early nutrition and of how critical early postnatal leptin levels are in the programming of feeding behaviour and energy use. It seems likely that some of these mechanisms were at work in our cohorts of animals, but this cannot be verified because we did not directly investigate the leptin surge or its neurotrophic consequences in the current study.

There is evidence from other studies to suggest that maternal insulin levels have programming effects on offspring. For example, hyperinsulinemia in rat dams is associated with obesity in offspring, in dams who received injections of insulin (516). It is a limitation of the current study that the F0 dams did not have plasma insulin and leptin levels assessed before mating or

monitored through pregnancy and lactation. It is possible that the MHF dams, raised on HF from weaning and significantly heavier than Control dams, were hyperglycemic and insulin resistant by the time of mating.

Dulloo (2008) proposes another programming mechanism to which our study data can lend support. He observes that the catch-up growth phenomenon is largely acquisition of adipose tissue, not lean: he calls this 'preferential catch-up fat' (517). He suggests that catch-up fat is the result of an energy conserving mechanism operating via suppressed thermogenesis, that is, a physiological down-regulation of energy expenditure. Although we did not directly measure energy expenditure in our animals, we did monitor energy intake, and can report that energy intake (kcals/g body weight) was unchanged from Controls across all groups. Since energy intake was equal but energy storage in the form of fat differed markedly, it follows that altered energy expenditure was the key factor in the equation of energy balance. Dulloo maintains that preferential catch-up fat is a wider phenomenon than developmental programming. It is seen also in other settings of weight recovery, such as in adult animals that have been starved and then returned to normal rations (518, 519), or in humans subjected to war-related famine or poverty (520, 521). Weight regained after nutritional deprivation in these other settings is typically preferentially fat over lean, and Dulloo suggests that the suppression of energy expenditure that that was adaptive during the state of deprivation has continued unrestrained after the food supply is returned to normal. Dulloo speculates that there are molecular links between catch-up adipose tissue expansion and restrained thermogenesis in skeletal muscle.

The current study confirms that there is altered energy expenditure in those cohorts born IUGR who then displayed catch-up growth. The growth trajectory of these cohorts led to increased adiposity in adulthood, yet with energy intake that was no different from Controls. This observation alone reveals the altered regulation of energy balance and fat deposition due to maternal dietary programming within these groups. Even when offspring were raised on a post-weaning diet of standard Chow, they gained significantly greater percentages of body weight as fat, compared to Controls, in both UNP and MHF/PLHF animals, in both males and females. Moreover, a post-weaning HF diet always exacerbated adiposity.

We did not make direct measures of energy expenditure in the current study, but other studies report that IUGR offspring of undernourished dams are significantly less active than Controls, irrespective of post-weaning diet (297).

This list of putative mechanisms underlying the developmental programming phenomenon is not exhaustive, and as yet the priority of any one mechanism over the others remains unclear. Several mechanisms may interact or several may operate in parallel, and this study was not designed to elucidate that question. Moreover, there are several critical windows where developmental trajectories may be malleable: we have suggested that maternal pre-conceptional body composition influences mRNA levels in adult offspring pancreas (the MHF group), and we have also demonstrated the power of postnatal catch-up nutrition, the prevention of which can rescue IUGR pups from subsequent metabolic syndrome symptoms in adult life (the UNPL group). Catch-up fat, or at least the mechanisms underlying its acquisition and deposition, are crucial to understanding the developmental programming phenomenon, because this particular period and type of fat growth influences adult phenotype and susceptibility to disease at least as much, if not more, than excess fat *per se*.

Catch-up growth following IUGR has been a common feature in the offspring of the diverse maternal malnutritions in the different branches of this study, as we have compared UNP with MHF/PLHF offspring. Catch-up growth may be a wider and more fundamental physiological reaction to growth retardation, given that it occurs in contexts other than developmental programming, and at ages when developmental plasticity is normally thought to be ended. Although catch-up was common to both MHF and PLHF offspring, they exhibited distinct mRNA expression patterns. It would have been interesting to include the UNP offspring in the gene expression studies, to examine what changes in intracellular signalling pathways were associated with that phenotype. It was a limitation of this project that only mRNA transcription levels were quantified, without measurement of translation of mRNA to protein. Levels of mRNA and coded protein do not always marry, because of regulation of translational and post-translational processes. This was evident in our study, for example, in the lack of correlation between the mRNA levels for the preproinsulin genes *versus* the plasma concentrations of the hormone. Due to logistical considerations and the size of this project, determination of protein levels to supplement the mRNA expression data will be performed at a later date.

There appear to be common growth patterns that are shared by the offspring of our very different maternal dietary treatments, and at first glance this is suggestive of a common mechanism leading from maternal malnutrition to adult offspring obesity and the metabolic syndrome. This is probably too simplistic an idea however, given the complexity of the physiology and the variety of proposed mechanisms. We ourselves have shown the unexpected result of two cohorts of offspring with very similar phenotypes and with two somewhat similar maternal HF dietary backgrounds – yet with very different mRNA gene expression profiles in essential intracellular

signalling systems. Nonetheless, a solid conclusion can be drawn as to the importance of maternal nutrition, of maternal body composition pre-pregnancy, of balanced nutrition through gestation and in the early postnatal period. These early stages of nutrition contribute powerfully to the risk of non-communicable disease later in the life course.

6.6 Limitations and future directions

The use of whole pancreatic tissue for gene expression studies was a limitation of this study. Endocrine tissue, which was the focus of our investigations, amounts to only 2% of the volume of the pancreas, with the remainder being exocrine tissue, blood vessels and ducts (522). Moreover, the endocrine islets of langerhans comprise of more than β-cells, and the other islet cells (such as the glucagon producing α-cells) may also have been expressing some of the mRNA species whose levels we measured in this study. It would have been preferable if we had been able to work solely on pancreatic islets as opposed to whole tissue. Isolated pancreatic islet techniques as utilised by Thompson *et al.* (405) or laser capture microdissection techniques (523) could make this possible. Notwithstanding this, it is believed that many of the mRNA levels we assayed are restricted within pancreata to the insulin-secreting β-cells: mRNAs such as insulin1 and 2, the insulin receptor, insulin receptor substrates 1 and 2, and the long form leptin receptor ObRb.

In the part of our study examining the effects of a maternal obesogenic diet on offspring, it would have been advantageous to include a group of dams raised on high fat from weaning, who were then returned to Chow before mating. This would have allowed us the opportunity to better examine the pre-conceptional effects of maternal obesity – especially since we have observed mRNA differences in key genes of insulin and leptin signalling pathways which may be due to maternal pre-conceptional adiposity. There would also be clinical relevance in the long term effects on offspring health produced by maternal weight loss before conception and pregnancy, especially given the known increased risks to the baby associated with obesity in humans. Zambrano *et al.* (2010) halted an obesogenic diet in female rats one month before mating and saw a partial reversal of metabolic syndrome signs in adult offspring (524). It would be interesting to use our model to investigate the effect of such a dietary manipulation on mRNA levels of key metabolic genes.

We also need to pursue histological studies on our islet tissues, to examine for changes in β-cell mass and morphology, to investigate functional changes such as hyperinsulinemia. Another essential next step in our pancreas work is to go beyond mRNA and look directly at protein levels. This would provide a more secure base to our findings of mRNA changes in key genes.

Changed levels of mRNA do not always translate into altered protein levels, because of post-transcriptional moderation of mRNA levels and of translational processes. Western blotting (protein immunoblot) methods could be utilised to explore end-levels of protein expression. Epigenetics is another direction for further work: whether early developmental exposure to malnutrition altered DNA methylation on any of the genes whose mRNA levels we have assayed.

The issue of energy balance, intake *versus* expenditure warrants further study. We have suggested that energy expenditure is the factor in the energy equation that is altered by early nutritional experience during critical windows. Moreover, adult female rats have a higher energy intake than males, regardless of programming. Basal metabolic, thermogenesis, and physical activity levels could all be investigated.

The studies detailed in this thesis utilised very large animal numbers. While this gave the benefit of statistical power to our work it also posed logistical difficulties and precluded the use of some more challenging techniques, such as taking blood pressures or conducting oral glucose tolerance tests. The size of our various cohorts has allowed us to generate an enormous amount of descriptive data, but the last part of this study, the gene expression work, has had to be restricted to the offspring of the obesogenic dams only, and then to just the chow-fed males. This has prevented us from probing for gender effects in this branch of the study, but has made the long and complex task of quantitative PCR procedures more manageable. Another aspect of this work worth investigating is the transgenerational effects of our maternal dietary treatments, but this also tends to logistical problems with amplification of animal numbers.

Food choice or preferences is another area worth exploring. We have shown in this current study that amount of food consumed differed little between groups. The underlying mechanism of this merits exploration: were calories the regulator? Or was it protein, whose levels were relatively constant across our diets? If rats were given an opportunity to choose the balance of carbohydrates *versus* fats *versus* protein in their diets, how would this balance be affected by developmental programming? Was the composition of the fat content of our diets important? Many diets have been used in developmental programming studies. We have demonstrated a powerful effect from the dietary treatments we have instigated in the current study and the models now need to be utilised with a more mechanistic focus. Some of the compositional elements (such as type of fat and omega 3:6 ratios) could be manipulated to further probe the mechanistic basis of the observed programming effects. An understanding of the underpinning mechanisms remains the goal of this area of research.

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