Maternal Dietary Restriction and the Effects of Postweaning Nutrition on Fetal Development, Insulin Signalling, Glucose Metabolism and Body Composition In C57BL/6J Mice

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Mice (C57BL/6J: B6) exposed to maternal dietary restriction (DR) exhibited fetal growth-restriction and as adults develop symptoms of the metabolic syndrome. We aimed to determine the impact of DR on fetal hepatic gluconeogenic pathway and insulin sensitivity in late gestation. Second, we aimed to determine whether a postweaning diet rich in omega-3 fatty acids would alter the development of glucose intolerance, insulin resistance and obesity in DR male offspring. The reduced rate of fetal glycogen synthesis by DR male offspring and altered hepatic gene expression of enzymes involved in insulin signalling and glucose metabolism suggest abnormal fetal development in response to DR that may contribute to the later development of the metabolic syndrome. The postweaning omega-3 diet improved obesity, glucose intolerance and insulin resistance in both DR and control males. These data suggest that nutrition in pregnancy and postnatal life play important roles in determining life-long metabolic health.
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Contributors

The following persons contributed to results presented in this thesis:

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Fetal measurements shown in Figure 2.3 were performed in collaboration with Richard Maganga

Chapter 3

Extraction of hepatic mRNA for gene expression measurement shown in Figures 3.3 and 3.4 were performed in collaboration with Anna Dorogin

Maternal and fetal measurements shown in Figure 3.5 and Table 3.3 were performed in collaboration with Dr. Kristin Connor and Elzbieta Matysiak-Zablocki

Extraction of hepatic protein for ELISA analysis shown in Figures 3.9 and 3.10 were performed in collaboration with Elzbieta Matysiak-Zablocki

Chapter 4

Weight measurements of male offspring shown in Figure 4.4 were performed by Dr. Brian Knight

Glucose tolerance testing shown in Figure 4.6 was performed by Dr. Brian Knight
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List of Abbreviations

11ß-HSD2 – 11ß-hydroxysteroid dehydrogenase 2

ω-3 FA – omega-3 fatty acids

CON – control

CVD – cardiovascular disease

DHA – Docosahexaenoic acid

DR – dietary restricted

GSK3 – glycogen synthase kinase-3

GWAS - genome-wide association studies

IR – insulin resistance

INSR – insulin receptor

IRS1 – insulin receptor substrate 1

IRS2 – insulin receptor substrate 2

IUGR – intrauterine growth restriction

PDK - phosphoinositide-dependent kinase

PI3K – phosphoinosital-3 kinase

SH2 - Src homology-2

T2D – type 2 diabetes

UDP-glucose – uridine diphosphate glucose

VLDL – very low density lipoproteins
Chapter 1: Introduction

1.1. The Metabolic Problem Associated With Diabetes and Cardiovascular Disease

The rising prevalence of diabetes and cardiovascular disease (CVD) places huge financial and medical burden not only on our national resources, but also our quality of life. By 2012, an estimated 2.8 million Canadians will be diagnosed with diabetes, numbers that are projected to increase further by 2020 when 10% of the population is estimated to be diabetic [1, 2]. The associated economic cost is staggering: $12.2 billion in 2010 alone, rising to a forecasted $16.9 billion by 2020 [1]. In addition, diabetes can contribute to the development of CVD [3], which is an even greater financial drain than diabetes and is the 2nd leading cause of death in Canada as of 2007 [4]. Both diabetes and CVD are chronic diseases that require careful monitoring and management, which may negatively affect individuals’ quality of life [5]. The full onset of type II diabetes (T2D) and CVD can co-exist and are preceded by a series of interrelated risk factors collectively referred to as the metabolic syndrome, which includes hypertension, insulin resistance, dysglycemia, dyslipidemia and abdominal obesity [6-8]. Currently, extensive research efforts aim to gain a better understanding of the causes and consequences of the metabolic syndrome and its risk factors, and to develop potential therapeutic targets and interventions to reduce the incidence of these diseases. The experiments described in this thesis will contribute to this understanding and will inform future studies in this field. This introduction will therefore shed light on the contribution of genetics and environment to fetal development and the later manifestation of metabolic disease.

1.1.1. Obesity

There is currently a global epidemic of obesity with the disorder being prevalent in approximately 24.1% of Canadians from 2007-2009 [9]. In obese individuals, adipose tissue contains abnormally elevated levels of triglycerides, which are released into circulation as free fatty acids with detrimental effects [10]. Body fat content can be measured to determine level of obesity using Dual-Energy X-ray Absorptiometry (DEXA) [11], however due to its high cost and
required methodological efforts, predictive measures of fat mass are often used instead and include body mass index (BMI) [12], waist circumference [12] and skinfold thickness [11]. Visceral obesity is an obese phenotype characterized by an increase in abdominal adipose tissue [13] that significantly contributes to the development of the metabolic syndrome [14]. Obesity may stem from an imbalance in energy homeostasis in which energy input is greater than expenditure [10]. If this is the case, then there is excess energy that may be stored as adipose tissue [10]. Some individuals who may transfer energy from food to fat and easily become obese, whereas others do not gain weight because more food energy is released as heat [15]. In other cases, genetics can disrupt this energy balance to impact one’s inclination towards higher body fat accumulation and this will be discussed later in this chapter. There exists a strong correlation between obesity and the development of the metabolic syndrome. In particulate, obesity is related to over 50% of T2D cases [16]. Childhood obesity is linked to the prevalence of the metabolic syndrome in adulthood and the incidence of metabolic syndrome increases with elevated childhood BMI [17]. Moreover, obesity may manifest with symptoms of the metabolic syndrome in children and adolescents, who are then at a greater risk of developing the full-onset of T2D and CVD in adulthood [18]. Obesity is a systemic, non-communicable disease that may manifest in several different ways and may increase the susceptibility of developing the metabolic syndrome. As such, the international epidemic of obesity has increased the need for understanding its underlying mechanisms.

1.1.2. Insulin Resistance

1.1.2.1. The Physiological Role of Insulin

Insulin is a hormone responsible for stimulating glucose uptake by peripheral tissues following an elevation of blood glucose levels, which occurs in the postprandial state [19]. Insulin regulates glucose metabolism and is stored in cytoplasmic secretory vesicles in pancreatic \( \beta \)-cells [15]. High blood glucose concentrations increase glucose transport into the \( \beta \)-cell, which triggers a series of events that result in exocytosis of the insulin-containing vesicles and hence, insulin secretion [15]. After entering the blood circulation, insulin stimulates glucose uptake in the liver and skeletal muscle, inhibits lipolysis in the adipose tissue, and inhibits gluconeogenesis and
glycogenolysis in the liver [14, 20, 21]. Food consumption or periods of fasting may be reflected by blood sugar concentrations, which regulate the levels of insulin secretion [19].

1.1.2.2. Insulin Regulation of Glycogen Synthesis

Insulin release initiates a signalling cascade that leads to glycogen synthesis [19, 22] (Figure 1.1). Insulin first encounters the insulin receptor (INSR), which consists of two α subunits located extracellularly that contain insulin binding sites, and two β subunits located intracellularly that contain the insulin-regulated tyrosine protein kinase [20]. Binding of insulin to the α subunit induces a conformational change that de-represses the kinase activity of the β subunit, leading to transphosphorylation of its own tyrosine residues [20, 22]. The β subunit subsequently phosphorylates tyrosine sites on insulin receptor substrates (IRS’s), such as IRS1 and IRS2 [22]. These substrates subsequently interact with various signal transduction proteins through their Src homology-2 (SH2) domains [22]. Following SH2 domain-binding, a major pathway of insulin signalling involves phosphoinositidal-3 kinase (PI3K), which becomes active once its SH2 domains are bound to tyrosine-phosphorylated IRS proteins [22]. Active PI3K phosphorylates phosphatidylinositol at the 3’-position to produce phosphoinositidal-3-phosphates that then recruit phosphoinositide-dependent kinases (PDKs) and another kinase called “Akt” or protein kinase B [22]. Once bound, phosphorylations by the PDKs activate Akt [23]. Akt then phosphorylates glycogen synthase kinase-3 (GSK3), preventing it from phosphorylating glycogen synthase and thus allowing glycogen synthase to act upon uridine diphosphate (UDP)-glucose to synthesize glycogen [22]. The regulation of these insulin signalling substrates may play a large role in insulin resistance and may be measurable in terms of hepatic glycogen production [24, 25]. Therefore, the insulin signalling pathway that leads to glycogen synthesis is of particular interest to our studies.
1.1.2.3. The Pathophysiology of Insulin Resistance

Insulin resistance (IR) is defined as the abnormal physiological state in which insulin concentrations are insufficient to adequately produce a normal insulin response in target tissues [26]. IR can occur through a number of ways, all of which are unified by a characteristic inhibition or decrease in insulin signalling [27, 28]. To compensate for tissue IR, pancreatic insulin secretion from β-cells increases, which results in compensatory hyperinsulinemia to lower blood glucose concentrations to normal levels [16, 29]. Eventually, overworked β-cells are unable to release the excess insulin required to achieve euglycemia, resulting in insufficient circulating insulin concentrations that cannot overcome increasing tissue IR [16]. This leads to hyperglycemia and overt T2D [14]. IR is a considerable risk factor in driving the processes underlying the metabolic syndrome [30].
1.1.3. Obesity and Insulin Resistance

Obesity and IR are intricately linked in several ways, with visceral obesity considered to be the major cause of IR [14, 21, 31]. The elevated circulating triglyceride levels often associated with an obese state lead to triglyceride uptake and accumulation in non-adipose tissues, including skeletal muscle and the liver [21]. In these tissues, triglycerides are metabolized into fatty acid intermediates that inhibit insulin signalling by negatively regulating insulin action, leading to IR [21]. Adipocytes also contribute to obesity-induced IR by secreting hormones and cytokines referred to as “adipokines,” including proinflammatory cytokines that suppress insulin signalling in adipose tissue and the liver [27, 32, 33]. The amount of adipokines secreted from adipocytes is relative to adipocyte size, which is itself proportional to the amount of stored triglyceride [16]. Hence, it appears that adipokines are direct mediators, whilst free fatty acids are indirect mediators of insulin resistance associated with obesity [16]. Studies in rodents have also found that obesity and high fat feeding promotes inflammatory pathways in the liver leading to IR and T2D [34]. Thus, targets for possible intervention for preventing the development of IR are the inflammatory pathways that interrupt the insulin signalling cascade leading to obesity-induced IR. Alternatively, IR can contribute to the development of obesity by influencing lipid metabolism and is believed to have a central role in the development of dyslipidemia [14].

Normally, insulin inhibits the production of VLDL (very low density lipoproteins) by the liver [14]. But in the insulin-resistant state, there is an overproduction of VLDL leading to increased circulating triglycerides, as seen with obesity [35]. Hence, obesity and IR may be studied in parallel, as they are closely related metabolic syndrome co-factors that may contribute to the progression of one another.

While obesity significantly increases the incidence of IR, it is not essential for the development of IR since IR individuals can be lean. A study by Petersen et al. (2006) compared lean, healthy young and elderly people and found that the elderly group had a tendency towards developing insulin resistance that was associated with increased fat accumulation in muscle and liver tissue [26]. These findings were believed to be a result of reduced mitochondrial function that lead to reduced mitochondrial oxidation of lipids and a subsequent increase in muscle and liver triglyceride content [26]. As well, insulin resistance has been observed in non-obese women
suffering from polycystic ovary syndrome (PCOS) [36, 37], which may be attributable to a genetic mutation that inhibits insulin signalling [36]. Therefore, IR is capable of manifesting in the absence of an obese phenotype.

Diet plays a significant role in the development of the metabolic syndrome. Today more than ever, many populations consume a greater number of daily calories than are required to sustain health and maintain adequate nutritional stores [38]. This is because of the reduction in the need for strenuous physical activity in the workplace and in the home, decreasing requirements for ingested energy, and the abundance, availability, palatability, and low cost of unhealthy foods [38, 39]. Increase in exercise and improved diet have concurrently showed improvement in risk factors included the metabolic syndrome, such as reduced central obesity and reduced fasting glucose [40]. Poor lifestyle and diet are important contributing factors to the development of the metabolic syndrome.

1.1.4. Genetics Associated with the Metabolic Syndrome

Genetic vulnerabilities may influence an individual’s likelihood of developing the metabolic syndrome. Studies on monozygotic twins reared separately, and on dizygotic twins raised together, have traditionally been used to study how environmental factors and genetics explain similarities/differences in the development of genetically-identical individuals [41]. Twin studies have found BMI to be highly heritable at an estimated 40-70% in children and adults [41-44]. Furthermore, T2D is also inherited to a great degree at approximately 20-58% in adult twins [45, 46]. In addition, population cohort studies have shown that the metabolic syndrome is largely heritable [47]. Genome-wide association studies (GWAS), which compare patient cases and control populations, aim to identify genetic variations or single nucleotide polymorphisms (SNPs), that are associated with disease [48]. GWAS have been highly productive in identifying genetic variants linked to the development of obesity; for example, a GWAS of T2D revealed that adults who were homozygous for the high-risk allele in the FTO (fat mass and obesity) gene had a greater body weight and a 1.67-fold increased odds of obesity than those who did not inherit a risk allele [49]. Future studies will potentially discover other genetic biomarkers for the heritability of the metabolic syndrome and further our understanding of the mechanisms
involved. The identification of such genetic biomarkers will enable screening and identification of individuals that have a greater risk for developing metabolic disease and may allow for early intervention in at-risk individuals. Moreover, the identification of genetic variants highly associated with the metabolic syndrome should also involve studying the role of these genes in developing disease. For instance, T2D is polygenic and is associated with at least 19 common alleles [50], which may involve polymorphisms in multiple genes encoding the proteins involved in the insulin signalling pathway [51]. Hence, genetics may contribute to the risk of developing the metabolic syndrome phenotype and studying the associated patterns of gene expression may increase our knowledge of the underlying mechanisms in this disease.

“Epigenetics” describes the study of patterns of genetic expression that are reversible or stable, and that can be meiotically or mitotically inherited [52]. In mammals, epigenetic changes are mediated by molecular mechanisms such as DNA methylation of cytosine residues on CpG islands, histone acetylation and microRNAs [53]. Epigenetic information may be influenced by the environment within a lifetime and then transmitted to the next generation [52, 54]. For example, a study in mice by Carone et al. (2010) demonstrated that a low protein, high-sucrose paternal diet consumed pre-conception increases DNA methylation in an intergenic locus region upstream of PPARα, a gene involved in regulating lipid synthesis. This was associated with an increase in hepatic expression of genes involved in lipid and cholesterol biosynthesis that may explain the decrease in cholesterol esters in the livers of low-protein offspring in comparison to offspring from males fed the control diet [55]. Moreover, maternal nutrition during pregnancy may influence fetal epigenetic regulation, leading to subtle alterations in gene expression of key metabolic genes and protein abundance that may prime the offspring for developing the metabolic syndrome [54, 56-58]. Rodent studies have shown that maternal nutrient restriction can reduce DNA methylation of the hepatic promoters of transcriptional regulators of key metabolic genes in glucose metabolism and adipogenesis, leading to altered gene expression, and that these epigenetic changes are found even in second generation progeny [59]. Hence, in addition to the genome, the epigenome may predict gene expression and physiology. Due to its plasticity during development, the epigenome may be an initial “sensor” of the early life
environment that changes molecular mechanisms in the fetus and that alters the health and disease phenotype of offspring in later life [60].

1.2. Developmental Origins of Health and Disease (DOHaD)

The maternal environment plays a large role in shaping fetal development and it may contribute to the eventual health and disease outcome in offspring [61-65]. One prime example of this is the cohort women affected by the Dutch famine of 1944-1945 and their adult children, a population that has been retrospectively studied to determine the effects of maternal dietary restriction on the development of offspring [61]. During the winter of 1944-1945, a population in the Netherlands had strictly rationed daily food provisions, which fell to below 1000 calories per day at its lowest and lead to severe malnutrition including in women at various stages of pregnancy [61]. Children who were \textit{in utero} during the famine during early gestation were born heavier, whereas those who were \textit{in utero} at mid- and late-gestation were born smaller and lighter. The difference in these observations points to how maternal undernutrition at various gestational periods may distinctly alter fetal growth. Those exposed to the famine from mid- to late gestation were also more likely to develop glucose intolerance [62]. Barker \textit{et al.} first reported an inverse relationship between small birth weight within the normal birthweight range and rates of mortality from coronary heart disease [63]. This study lead to the establishment of the “fetal origins” hypothesis whereby events \textit{in utero} lead to fetal adaptive development that permanently alters the physiology of the offspring such that the risk of developing heart disease and diabetes in later life is increased [66]. These offspring exposed to poor intra-uterine conditions are effectively “programmed” to develop the metabolic syndrome in later life [67]. Since then, both human and animal studies have found similar associations between birth size and the incidence of the metabolic syndrome in adulthood, including hypertension, stroke, insulin resistance, type 2 diabetes and dyslipidemia [63-65]. Symptoms of the metabolic syndrome may be attributable to poor fetal nutrition in early life. This association lead to Hales and Barker proposing the “thrifty phenotype hypothesis,” which suggests that the intra-uterine environment acts as an indicator of the maternal environment for the developing fetus [68]. If there is a low intrauterine supply of nutrients, then the fetus may adapt by reducing somatic growth in order divert limited nutrients to essential developing tissues and its metabolism may be altered to compensate by increasing
energy production in preparation for postnatal life [66, 68, 69]. However, fetal adaptations to a poor nutrient environment may be maintained postnatally, even if the offspring is born into a nutrient-rich environment [69]. The mismatch between predicted adaptive responses to poor fetal environment and the nutrient-rich postnatal environment may then lead to offspring developing symptoms of the metabolic syndrome, such as glucose intolerance, insulin resistance and obesity [64, 70]. These studies and hypotheses have lead to the establishment of the Developmental Origins of Health and Disease (DOHaD) hypothesis, which states that factors contributing to suboptimal conditions and leading to abnormal fetal development in early life (pregnancy and infancy) may increase the individual’s risk of adulthood disease [71]. As seen in the Dutch Famine cohort, nutrition is one maternal environmental factor that contributes to programming because it has a significant impact on fetal growth [61-63]. Maternal nutrition may determine whether the fetus has an optimal environment for development and will be discussed later in this chapter.

1.2.1. Glucocorticoids Programming of Metabolic Disease

Increased fetal exposure to glucocorticoids is believed to be one of the major causes of the elevated risk for the metabolic syndrome [72]. Glucocorticoids are synthesized in the adrenal cortex as part of the hypothalamic-pituitary-adrenal (HPA) axis [73, 74]. Glucocorticoids mediate the “Fight-or-Flight” response to stressful insults, and their actions include increasing hepatic gluconeogenesis, elevating blood pressure, suppression of inflammation, initiating parturition, suppression of immune reactions, and inhibition of glucose uptake in peripheral tissues [72, 73, 75]. Glucocorticoids are important for fetal tissue maturation [76] with most early embryonic tissues expressing glucocorticoid receptors [77, 78]. Maternal undernutrition and placental insufficiency lead to increased maternal and fetal glucocorticoid levels in circulation in rodents, sheep and humans, and are associated with reduced fetal body weights [76, 79-81]. Increased glucocorticoids in fetal circulation following maternal undernutrition occur through two known mechanisms. First, in response to maternal undernutrition there is downregulated expression of placental 11β-hydroxysteroid dehydrogenase (HSD)-2, which converts C11-hydroxylated corticosteroids (cortisol, corticosterone) into their inactive C11-keto metabolite forms (cortisone, 11-dehydrocorticosterone) in mammalian tissues [82]. Placental
11β-HSD2 therefore protects the fetus from overexposure to the much higher maternal glucocorticoid levels [83]. In rats, offspring of dams fed a low protein diet for the duration of pregnancy have reduced placental 11β-HSD2 expression [84]. We have also observed that maternal caloric restriction reduces placental 11β-HSD2 and is associated with an increase in fetal corticosterone levels in C57BL/6J mice [85]. Thus, in the absence of or a reduction in the placental 11β-HSD2 barrier there may be an increase in active maternal glucocorticoids transferred into the fetal bloodstream. Second, maternal undernutrition is associated with an increase in maternal production of glucocorticoids in both humans and rats [86, 87]. Together, the lack of inactivation of corticosteroids and increased flux towards the fetus from maternal circulation significantly raises fetal exposure to glucocorticoids may impair fetal development. Increased fetal exposure to glucocorticoids prematurely stimulates the HPA axis activity, which can alter tissue function and physiology during critical windows of development, induce fetal growth restriction [88], dysregulate HPA axis function in adulthood, and may significantly raise offspring risk of developing disease in later life [72, 87, 89]. Hence, fetal glucocorticoid exposure is one of the major underlying mechanisms of maternal undernutrition leading to the increased risk of the metabolic syndrome in adulthood.

1.3. Maternal Undernutrition

Maternal nutrition may largely determine fetal nutrition [90], which supports maternal undernutrition during pregnancy as an important area of study for several reasons. First, there are developing nations where proper nutrition is not readily accessible to pregnant women. Second, in developed societies, women may consciously restrict their caloric intake during pregnancy to maintain their ideal body image [91]. Third, a woman may accidentally consume fewer calories than what is needed in early gestation because she has not yet realized that she is pregnant. It would be important for her to know how to improve her nutrition during the remainder of her pregnancy for the best possible health outcome for her child. Maternal undernutrition during pregnancy is found on an international level and needs to be further investigated because it is strongly associated with offspring developing the metabolic syndrome [7, 61, 62]. Animal models have been useful for investigating the underlying mechanisms of maternal undernutrition because they allow for more invasive studies that cannot be carried out in humans.
There are various animal models of maternal undernutrition and a range of species have been utilised, with sheep and rodents being the most predominant ones [92-95]. For example, sheep have been widely used in the study of the impact of maternal undernutrition on fetal growth [96], because this large animal model allows for the easy insertion of physiological measuring devices, the increased amount of tissue available, and evidence that observations in this species are highly applicable to humans [97]. However, maintenance of ovine cohorts requires large areas to hold animals and animal surgeries are expensive, which have lead researchers to increasingly turn to rodent models [97]. Sheep are also phylogenetically distinct from primates and differ in placentation compared to humans [98]. Rodents are often used for animal models of disease because of their availability, short gestational age, large litter sizes, ease of handling, use for “knock-out” models, requirement for less space, use for generational studies, and because their genomes are highly similar to that of humans [99, 100]. Structural and functional similarities of organs between rodents and humans have lead to the use of rodents to study the development of organs, such the liver [101]. However, disadvantages of the rodents include their small size, which may prevent cannulation of the fetus and make physiological assessment difficult [95, 100]. In addition, mice, rats and rabbits, give birth to relatively immature newborns compared to humans. For example, by birth, humans are neuronally advanced compared to mice, which have a significant portion of their neural development taking place postnatally [102]. Also, there are differences between rodent and human placentation in terms of structure, function and development [98]. Analysis of mouse and human embryonic development has revealed spatial and temporal differences in the expression of genes involved in development and disease [103]. While there is no model capable of replicating the human situation, however, due to the aforementioned reasons and for ethical reasons, mice are an appropriate alternative species to study maternal undernutrition for the purposes of this thesis.

Animal studies of maternal undernutrition often differ in the type of dietary restriction that is imposed. Some studies implement a low-protein diet while others may use a global caloric restriction during pregnancy [68, 85, 87, 104-106]. Studies also vary in the severity of the dietary restriction. For example, reductions in caloric intake in some studies may vary between 30% [85, 106, 107] to 70% [108] of ad libitum intake. Both maternal global undernutrition [109-112] and
protein restriction [113] in rodents have induced obesity and metabolic disorders in rodent offspring during adulthood. These two types of diets may exert their effects on fetal development through different mechanisms. Dumortier et al. (2007) showed that both a maternal protein restriction and a 50% maternal caloric restriction reduced fetal pancreatic β-cell mass in rats through different mechanisms [114]. The maternal low-protein diet decreased islet vascularization that may account for the lowered β-cell proliferation. In comparison, maternal caloric restriction elevated corticosterone levels, which may have lead to the decrease in β-cell differentiation [114]. Thus, while both types of maternal undernutrition may lead to similar results in terms of metabolic disease, it is clear that each may do so through unique mechanisms and should not be considered interchangeable. Maternal protein-restriction is highly applicable when considering underdeveloped countries [108]. However, it is global caloric restriction that is perhaps more valid for Western populations in which socioeconomic status or health trends may reduce overall maternal intake of protein and other nutrients [108]. While both types of maternal undernutrition are valid, the focus of my studies is on the effects of global caloric restriction during pregnancy.

Animal models also differ in the timing of maternal nutrient restriction during gestation, which can exert specific effects on offspring as they age [70, 115, 116]. For example, 50% caloric restriction during late gestation in sheep leads to glucose intolerance and insulin resistance in adult offspring compared to controls, but offspring from ewes fed the same restriction during early gestation had similar glucose-insulin homeostasis when compared to control offspring [92]. These findings are similar to those of the Dutch famine cohort, in which maternal undernutrition in late gestation increased the risk of glucose intolerance in offspring [62]. The period of gestation during which maternal undernutrition is implemented may play a large role in determining the metabolic disease outcome in offspring and should therefore be taken into serious consideration when interpreting studies in animal models.

Fetal tissues mature at different rates and stages during gestation, and each have their own critical windows of development [116]. The difference in metabolic disease outcome observed in response to maternal undernutrition may be due, in part, to altered organogenesis in terms of
Animal models have provided insight into the critical windows of development for various organs. For instance, a 50% protein restriction from day (d) 10-20 of gestation reduced placental weight:fetal weight ratio in rats [117, 119]. Alternatively, a 50% protein restriction from d14-21 does not affect placental weight [120], but fetal weights were reduced, which also translates into increased placental weight-body weight ratios (although the authors did not calculate this). Similarly, placental weights were also reduced in dams fed 30% of ad libitum intake throughout pregnancy compared to control dams [112]. These placental studies in rodents suggest that the fetal placental weight ratio is sensitive to the maternal 50% caloric restriction up to d14, after which there is limited effect. Timing of maternal undernutrition also affects pancreatic development, as protein restriction during the last week of gestation decreased fetal β-cell mass to a greater degree than when it was imposed throughout gestation in rats [114]. A maternal low-protein diet also reduced postnatal pancreatic islet number, size and β-cell complement in rats, and the severity of impact varied according to the discrete gestational period during which the diet was fed [118]. A 50% maternal caloric restriction during late gestation also impaired β-cell development in offspring [121]. The differences in how organs develop in response to maternal undernutrition at various stages of pregnancy in rodents have revealed their critical windows of development.

1.4. The Liver: Development, Maternal Undernutrition and the Metabolic Syndrome

1.4.1. Liver Development and Function

The liver is an essential, insulin-sensitive organ that has several functions as it plays a key role in the regulation of whole body metabolism, including carbohydrate, lipid and protein metabolism [14]. Liver cells (hepatocytes) are the functional unit of the liver, and contribute to the conversion of ammonia to urea, production and excretion of bile lipogenesis, cholesterol synthesis, and coagulating factor synthesis [14, 27]. In particular, hepatocytes maintain blood glucose levels within a narrow range by regulating two major metabolic pathways, gluconeogenesis and glycogenolysis [27, 122]. When blood glucose levels are elevated, such as following a meal, the liver takes up and stores glucose in the form of glycogen to be released
during states of low blood glucose [123]. Hepatic glucose metabolism is controlled by circulating
hormones, namely insulin, which determine whether the liver is synthesizing or releasing glucose
into blood circulation [14].

Mice have been used to study fetal liver development because their hepatic structure and
physiological function is similar to humans [101, 124]. In mice, gastrulation occurs around d6.5-
7.5, leading to the formation of the endoderm, which subsequently divides into the foregut,
midgut and hindgut regions by d8.5 [101]. Then by d8.5, the hepatic specific portion of the
ventral foregut endoderm develops, with the budding of thickened endothelium developing into
the liver diverticulum by d9.0 [101]. The liver and intrahepatic biliary tree develop from the
anterior of the liver diverticulum, and the gall bladder and extrahepatic bile ducts form from the
posterior portion [101]. From d10-15, the liver undergoes accelerated growth and
vascularization, and becomes the major site of fetal haematopoiesis [101]. By d15, hepatoblasts
are maturing into hepatocyte and biliary cells and the liver continues to mature postnatally [101].
Liver mass continues to increase postnatally up to three weeks of age and then declines in the
fourth week [125]. While humans and mice have similar liver structure, comparison of the liver
transcriptome has shown significant differences in gene expression [124]. This suggests that the
two species regulate hepatic gene expression in dissimilar ways [124], however more research is
required to determine if these findings translate into differences in hepatic function

1.4.2. Maternal Undernutrition Alters Liver Development

Hepatic structural development and gene expression are affected by maternal undernutrition [95,
126]. First, maternal dietary restriction may alter physiological structure. For example,
Vonnahme et al. (2003) found that maternal 50% caloric restriction from early to mid gestation
increased liver weight per unit body weight in mid-gestation sheep fetuses [127]. Second,
maternal undernutrition leads to abnormal fetal hepatic gene expression. Whorwood et al. (2001)
used a similar maternal dietary protocol as Vonnahme et al. (2003) [127], and found that dietary-
restricted fetal sheep had altered expression of genes involved in glucocorticoid metabolism and
blood pressure regulation compared to control fetuses [128]. While it is difficult to obtain fetal
hepatic measurements in humans, low maternal pregnancy weight gain results in preferred
venous blood supply to the left lobe, at the expense of the right lobe, of the fetal liver [129]. Maternal undernutrition leads to hepatic lobar differences perhaps as a result of the difference in blood circulation. For example, a maternal low-protein diet fed throughout pregnancy and lactation leads to altered gene expression between the left and right liver lobes in adult male rat offspring [130]. Increased fetal hepatic gluconeogenic enzyme gene expression and activity is also observed in rat fetuses of dams fasted in late gestation compared to controls [131]. These gene transcription differences may program offspring to adapt to poor nutrient conditions, postnatally. However, if these offspring are born into a nutrient-rich environment, then there may be a mismatch of predictive and actual postnatal environments that leads to the development of metabolic problems in later life [67]. Maternal protein restriction in rats from d10 to the end of gestation increased adult hepatic gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression and elevated glucose concentrations compared to controls [132]. Additionally, maternal protein reduction throughout pregnancy in rats lead to higher PEPCK enzymatic activity in adult male offspring compared to controls [133]. Third, the impact of maternal undernutrition on fetal liver structure depends on the timing of dietary restriction; in sheep, fetal liver mass increased by maternal nutrient restriction from early-mid gestation [127], but it is reduced by maternal caloric restriction during late gestation only [134].

These findings suggest that maternal undernutrition results in abnormal fetal liver development that mediates the offsprings’ progression toward the metabolic syndrome. Supporting this theory, the liver is known to play a large role in the pathophysiology of metabolic disease and it has been proposed that hepatic insulin resistance is the most important force behind the metabolic syndrome and its associated conditions including hyperglycemia, dyslipidemia and increased inflammatory factors [14]. Thus, maternal undernutrition may lead to abnormal fetal liver development that predisposes the offspring toward the metabolic disease phenotype when faced with a nutrient-rich postnatal environment.

1.4.3. Maternal Undernutrition: The Next Generation

Studying how maternal undernutrition alters fetal development is highly relevant to healthcare today because induction of a metabolic syndrome phenotype is not only apparent in first
generation offspring but, can be inherited to some degree by future descendants. Women who suffered psychological stress and nutrient restriction during the Dutch famine gave birth to daughters with low birth weight and increased incidence of insulin resistance [135] and the subsequent generation of daughters also had low birth weights [136]. That maternal undernutrition may have negative repercussions for subsequent generations has far-reaching implications and makes understanding the mechanism of fetal programming that much more pertinent.

1.4.4. Sex Differences

Whether it be protein restriction [137], overnutrition [138], placental insufficiency [139], or caloric restriction [106] in animals or human models of altered maternal nutrition, male offspring are more likely to develop metabolic syndrome symptoms compared to females. It appears that offspring respond to changes in maternal nutrition in a sex-specific manner. In a study by McMullen et al. [140], all offspring exposed to low-protein diets throughout pregnancy had elevated systolic blood pressure, where the mechanisms underlying this phenotype were glucocorticoid-dependent in males and glucocorticoid-independent in females. The differences between gender responses may be a result of differences in sex hormone concentrations between males and females [139, 140]. In humans, circulating testosterone levels in men appear to influence the incidence of metabolic syndrome [141]. Furthermore, female hormones may have a protective effect against fetal programming of disease. In a rat model of placental insufficiency, both male and female pre-pubertal offspring had elevated blood pressure, however post-puberty males remained hypertensive while female BP normalized [139]. It appears that males are more vulnerable to the adverse effects of maternal undernutrition in a way that increases their susceptibility to developing the metabolic syndrome. Hence, it is important to look at the sexes separately when studying the effects of maternal nutrition on fetal development.

1.5. Interventions to prevent developmental programming of disease

Intervention strategies to reduce the incidence of non-communicable diseases must be found to prevent their financial burden on international health care systems and improve the quality of
life. A non-invasive approach is preferable and nutritional intervention is an ideal approach to lowering the risk of developing metabolic and chronic diseases. Nutritional studies compare the dietary composition of individuals with high and low metabolic syndrome to help identify foods that may increase or decrease the risk of developing metabolic disease. For example, a study by McKeown et al., (2004) observed that an increase in dietary intake of whole-grain foods was associated with a lower incidence of IR in middle-aged men and women [142]. Alternatively, cohort studies have compared minority populations that have lower metabolic syndrome rates compared to Western populations and found key differences in diet, including higher consumption of omega-3 (ω-3) fatty acids [143]. Together these studies may shed light on foods that can protect individuals from developing the metabolic syndrome. Intervening earlier, prior to the onset of disease, may increase the likeliness of preventing the development of metabolic disease with advancing age.

In the case of maternal undernutrition, a decline in access to essential nutrients negatively affects fetal development. An article by Bhutta et al. (2008) reviewed nutrition-related preventative interventions that could improve survival in mothers and stunted growth by children in 36 countries [144]. Approximately 10% of global deaths in children younger than 5 years of age are due to micronutrient deficiencies, with the majority being a result of vitamin A and zinc deficiencies [144]. Vitamin A and zinc supplementations were core recommendations based on the evidence showing the association with reduced stunted growth by children [144]. Iron supplementation during either pregnancy or infancy was also recommended as it helped reduce anaemia [144]. Fortified food by iodosation of salt or water provided to pregnant mothers reduced the incidence of goitre and subsequent neonatal mortality rates [144]. Furthermore, balancing energy and protein supplementation to expectant mothers reduced IUGR by over a third [144]. ω-3 fatty acids are essential for fetal brain development [145] and may be beneficial in pregnancy in several ways. Increased ingestion of docosahexaenoic acid (DHA), an ω-3 fatty acid, during pregnancy and lactation has been found to improve cognitive development of infants [146]. Interestingly, oral administration of DHA to pregnant mice increased surfactant production and secretion in the lungs of preterm fetuses [147]. This suggests the potential use of DHA to stimulate lung maturation in preterm newborn infants that may increase their survival
Some also suggested that maternal intake of ω-3 fatty acids should be increased to reduce preterm birth in high-risk pregnancies since they have been shown to extend gestation in humans and increase birthweight [148-150]. Global recognition of the large role maternal health plays in fetal development has resulted in international push towards improving the health of all women of reproductive age, and perhaps earlier [151]. The Centre for Disease Control (CDC) has issued recommendations and objectives towards improving periconceptual maternal health [152], while the United Nations has set one of the Millenium Development Goals as improving maternal health in developing countries [153]. These organizations have realized that targeting women and girls to receive adequate nutrition and health care will benefit them as individuals, their offspring in the long-term, and the health and well-being of populations. These efforts to better women’s health will help prevent offspring from developing the metabolic syndrome phenotype.

1.6. Rationale and Hypothesis

1.6.1. Rationale

Maternal undernutrition results in small-for-gestation or growth-restricted newborns with a higher risk of developing the metabolic syndrome, including impaired glucose tolerance, insulin resistance and obesity in later life [62, 64, 66-68, 154-158]. Poor maternal nutrition alters fetal metabolism, including the upregulation of hepatic gluconeogenesis [131]. These fetal adaptations, which occur during critical windows of development, may be maintained postnatally and influence an individual’s physiology and metabolism long-term. In rodents, the elevated fetal hepatic glucose production induced by maternal undernutrition may be maintained into adulthood, leading to increased gluconeogenic enzymatic activity and expression and hyperglycemia [88, 132, 133, 159]. Although some mechanisms through which maternal undernutrition leads to compromised metabolic phenotypes in offspring have been investigated, how growth-restriction following maternal undernutrition predisposes the offspring to developing metabolic disease in later life remains unclear.

The liver plays a major role in the development of metabolic disease and it has been proposed that hepatic insulin resistance is the most important driving force behind the metabolic syndrome [14]. The liver is an insulin-sensitive organ and maintains blood glucose levels within a narrow
range by regulating two major metabolic pathways, gluconeogenesis and glycogenolysis [27, 122]. The impact of maternal undernutrition on the development of fetal hepatic glucose control and insulin sensitivity requires more understanding and may contribute to the offspring’s increased susceptibility towards the metabolic syndrome.

Through dietary means there may exist non-invasive interventions to prevent the development of obesity and insulin resistance, and higher consumption of ω-3 fatty acids (FA) shows potential therapeutic benefit. Increased dietary intake of ω-3 FA has been shown to reduce the incidence of metabolic syndrome in men [160], and improve diabetes and cardiovascular risk factors in overweight, hypertensive, and hyperinsulinemic patients [161-163]. Therefore, considering the improvement in metabolic parameters in adults ingesting ω-3 FA, increased dietary ω-3 FA intake in postnatal life may reduce or inhibit the trajectory toward metabolic disease in offspring of mothers undernourished during pregnancy.

Previously we established a mouse model of moderate maternal undernutrition from mid-late gestation using the C57BL/6J (B6) strain of mice. Offspring of these dietary restricted mothers are growth-restricted in late fetal life, with males later developing indications of insulin resistance, glucose intolerance and obesity [85, 106]. Here we have a unique opportunity to study the mechanisms underlying the fetal adaptive response to maternal undernutrition that lead to development of the metabolic syndrome, and to intervene in early postnatal life to try and attenuate the development of metabolic disease.

1.6.2. Hypothesis

We hypothesized that maternal dietary restriction would result in growth-restricted fetuses and alter fetal liver development in late gestation, and a dietary intervention in postweaning life would lessen the adverse effects of maternal undernutrition on the metabolic phenotype of the offspring in adulthood.

Specifically, we hypothesized that a moderate 30% maternal caloric restriction from mid-late gestation would alter liver glucose metabolism and insulin signalling pathways in the B6 mouse fetus in late gestation and these changes may influence the onset of metabolic disease in later
life. We aimed to determine the effect of maternal dietary restriction on fetal hepatic glycogen synthesis, a marker for insulin signalling, and on expression of key genes involved in hepatic glucose and glycogen metabolism, in control and dietary restricted fetuses. Further, we aimed to determine the fetal hepatic insulin signalling response to insulin exposure following maternal dietary restriction.

Additionally, we hypothesized that increased dietary intake of ω-3 fatty acids in postweaning life would prevent the development or lessen the severity of the metabolic syndrome phenotype observed in adult offspring undernourished in utero. We aimed to determine the effects of an ω-3-rich postweaning diet from 3 months to 12 months of age on body composition, insulin sensitivity and glucose tolerance in male offspring of control fed and dietary restricted mothers.
Chapter 2
Establishing the Dietary Restriction Protocol

2.1. Introduction

Epidemiological studies have identified an association between low birthweight and an increased risk of developing cardiovascular and metabolic disease [61, 63-65]. Babies who are of low birthweight may be small for gestational age (SGA; birthweight below the 10\textsuperscript{th} percentile) and/or intrauterine growth restriction (IUGR; fetal weight below the 10\textsuperscript{th} percentile for gestational age, abdominal circumference below the 2.5\textsuperscript{th} percentile, and birth length is 2 standard deviations or less below the mean for gestational age) [164-166]. SGA newborns may be genetically-predetermined to be small, but they are otherwise normal [165]. In comparison, IUGR newborns, for a variety of reasons, fail to reach their growth potential [165]. Causes of IUGR include placental insufficiency [167], abnormal placental development [167], and altered maternal nutrition [168]. IUGR may affects as many as 5\% of all human pregnancies [168], and is associated with a higher incidence of preterm birth [169]. IUGR has been associated with increased incidence of anxiety [170], ischemic heart disease [156], insulin resistance and metabolic disorders [168] in adulthood. Thus, IUGR offspring are an important clinical population to study so that we may further our understanding of the underlying causes of IUGR with the aim to prevent and treat this disorder to improve the health of these individuals both in the short and long term.

Insulin and leptin are hormones with major roles in regulating fetal growth and development and are implicated in modifying the risk of disease in later life. In the human fetus, β-cells in the pancreas differentiate at d26 and pancreatic insulin synthesis is apparent by gestational d33; pancreatic islets assemble by 12-13 weeks of gestation [171]. In mice, pancreatic β-cells begin to mature and begin to secrete insulin in quick succession from approximately day (d) 9.5 to 10.5 of gestation in mice while islet formation occurs near term [171]. In adults, insulin is secreted by the pancreas in response to elevated blood glucose concentrations, stimulating glucose uptake and glycogenesis, and inhibiting gluconeogenesis [21]. In fetal life, insulin is also glucose-
regulated and has a major role in promoting tissue accretion [86], with a lack of insulin secretion lowering birthweight up to 20-33% in mice and humans [172-176]. Insulin concentrations in cord blood from IUGR newborns is similar to [177, 178] or lower than [179] concentrations in newborns of normal birthweight, which may be a consequence of reduced pancreatic β-cell mass [180]. This trend is also seen in the IUGR sheep and rodent fetus, associated with reduced pancreas weight [80, 94, 181]. It is theorized that IUGR offspring are unable to secrete sufficient insulin to maintain normal glucose metabolism in postnatal life and thus eventually develop glucose intolerance [182]. Reduced pancreatic insulin content in IUGR fetuses may result from lowered nutrient supply in both humans and rodents [121, 182, 183].

Adipocyte production of leptin can be stimulated by insulin, and in turn initiates the hypothalamic-derived satiety signal to decrease food intake and stabilize energy metabolism [184, 185]. IUGR newborns have low cord blood concentrations of leptin compared to newborns within the normal birthweight range, which correlates with reduced fetal fat mass, increased leptin resistance and obesity in adulthood [186, 187]. Low fetal leptin expression levels in the circulation may reduce the ability to regulate food intake and energy expenditure demands in later life [187]. Attig et al. [188] subcutaneously administered leptin receptor antagonists to rats from postpartum day 2-13. After being placed on a high fat diet, these offspring developed leptin resistance and obesity at 4 months of age [188]. The days following parturition are still critical periods of development for rodents that are equivalent to prenatal development in humans. If the study by Attig et al. [188] could be related to human populations, their methods might be equivalent to decreasing leptin sensitivity in the late gestation human fetus, which might reflect the effect of low fetal leptin levels. Additionally, a study by Bouret et al. demonstrated that the hypothalamic development that occurs during the leptin surge in late gestation is altered in leptin-deficient mice but can be regained by treating neonates with exogenous leptin [189]. There are comparable increases in maternal leptin levels with advancing gestation in humans and rodents [190-192] In humans, maternal leptin is largely derived from the placenta [107, 193]. While the placenta also contributes to fetal leptin levels [186, 194], fetal leptin synthesis and secretion increases as fetal adipose tissue develops in late gestation [186, 195]. In rodents, leptin levels in the maternal blood rise during pregnancy, but the placenta is not a major source of
leptin for fetuses [192, 196]. In rat fetuses, leptin originates from the transplacental passage of maternal leptin [197]. Maternal undernutrition reduces fetal fat mass, which lowers leptin secretion. Low fetal leptin levels are associated with IUGR and impaired hypothalamic development that leads to the inability to regulate food intake in later life.

In some cases of IUGR, the initial cause of low birthweight can be attributed to reduced maternal nutrition during pregnancy. In rodents, maternal caloric restriction imposed at various stages of gestation results in growth-restricted offspring that develop the metabolic syndrome in later life [68, 87, 93, 94, 105, 110, 198-200]. Our lab has previously developed a model of maternal undernutrition using C57BL/6J (B6) mice that results in an increased incidence of preterm birth, and reduced offspring birthweight, associated with obesity and glucose intolerance in adulthood [85, 106]. Recently, our research programme relocated to a new animal facility requiring us to re-establish our developmental programming mouse model. Hence, we hypothesized that a moderate 30% caloric dietary restriction (DR) imposed on pregnant B6 mice from mid- to late-gestation, would produce IUGR fetuses, consistent with our previously published results, associated with altered concentrations of serum leptin and insulin.

2.2. Methods

2.2.1 Animals and Diet

All experiments were approved by the Samuel Lunenfeld Research Institute Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. Inbred, nulliparous C57BL/6J (B6) female mice (Jackson, Bar Harbor, ME, USA) were fed sterile 1 g Dustless Precision Pellets® (Bio-Serv, Frenchtown, NJ, USA) and sterile water ad libitum for at least 1 week prior to experimentation. The mice were housed under standard environmental conditions (12 hour light:dark cycle) in polysulfone microisolator cages with woodchip bedding and sterile cardboard nestlets. The Dustless Precision Pellets are a grain-based formula composed of 20% protein, 4% fat, and 55% carbohydrate, with the remaining percentage a composite of moisture, fibre, and ash (http://www.bio-serv.com/Rodent_DPPS/DPP_RGB.html). At 6-8 weeks of age, nulliparous female B6 mice were mated with an experienced male of the same strain. Two females and 1 male were placed in the male’s cage for up to 5 days with daily
monitoring for a vaginal sperm plug. The day a vaginal sperm plug was observed was designated as day (d) 0.5 of gestation. After d0.5 female mice were housed individually and fed 1 g pellets and water *ad libitum*. Food intake and body weights of the control (CON; n=14) pregnant females were measured 2-3 times per week. The average daily *ad libitum* food intake of CON pregnant females was calculated for each day, measured from d5.5 to d18.5. Dams with litters fewer than 6 or greater than 12 pups were excluded. Two CON litters were excluded (n=3 fetuses, n=1 fetus). Food-restricted (DR; n=10) B6 pregnant females were fed 30% of the average daily CON *ad libitum* food intake from d5.5 until killing at d18.5. Other studies have used similar or more severe caloric restriction during gestation. These also produce IUGR fetuses and led to the development of obesity, hyperinsulinemia, hypertension and hyperleptinemia in adulthood [87, 108, 110, 198, 201]. We selected a 30% caloric restriction for our study because while it is a relatively moderate challenge it is nevertheless sufficient to programme symptoms of the metabolic disease [106, 108].

At d18.5, dams were euthanized by cervical dislocation. Fetuses were extracted, anesthetized on ice, weighed and euthanized by decapitation. Maternal and fetal trunk blood was collected in Drummond EDTA-coated microcapillary tubes (VWR, Radnor, PA), allowed to clot on ice in Axygen Scientific 1.5mL eppendorf tubes (VWR, Radnor, PA), centrifuged at 13000 rpm 4°C for 15 min, and the resulting serum was stored at -80°C. Fetal heads, livers, placentae and adrenal glands were collected, weighed, flash frozen in liquid nitrogen and stored at -80°C.

2.2.2 Blood Serum Assays

Maternal and fetal serum glucose was measured using the One Touch Ultra Glucometer (LifeScan Canada Ltd, Burnaby, BC). Maternal and fetal serum was analysed for insulin and leptin concentrations using the enzyme-linked immunosorbent assay (ELISA) method. The following assays were used: insulin ELISA (ALPCO Diagnostics, Salem, NH) with an inter-assay coefficient of variation of 26.4% and an intra-assay coefficient of variation of 5.3%, and leptin ELISA (Crystal Chem Inc., Downers Grove, Il) with an inter-assay coefficient of variation of 87.3% and an intra-assay coefficient of variation of 1.9%. The limit of detection was 0.115
ng/mL for insulin, and 0.2 ng/mL for leptin. Due to small sample sizes, female fetal serum had to be pooled within each litter in order to measure serum insulin and leptin concentration.

2.2.3 Statistical Analysis

Data are presented as means ± standard errors of the mean (SEM). Number of litters (biological replicates) is indicated on the histograms. Statistical analyses were performed using JMP 9.0.2 (SAS Institute Inc, Cary, NC). Data were assessed for normality using the Kolmogorov-Smirnov test. Data were analyzed by Student’s t test with maternal diet as the factor. The influence of sex differences on the data was determined by two-way ANOVA, followed by post-hoc Tukey univariate tests, with maternal diet and sex as the factors. Data that was not normally distributed even after transformation were analyzed by Wilcoxon test. The repeated measures one-way ANOVA, followed by post-hoc Tukey univariate tests, was used to evaluate the change in maternal weight and dietary intake over time with maternal diet and time as the factors. P values less than 0.05 were considered statistically significant.

2.3. Results

2.3.1 Mothers

From d5.5 to d18.5, CON dams increased their daily food intake (28% increase; p<0.05, Figure 2.1A) and bodyweight (60% increase; unadj Epsilon, p<0.0001; p<0.01, Figure 2.1B). At d18.5, dietary restriction was associated with reduced concentrations of serum glucose (p<0.0001, Figure 2.2A), serum insulin (p<0.05, Figure 2.2B) and a significantly lower glucose:insulin ratio compared to control ad libitum fed dams (p<0.05; Figure 2.3C). Maternal serum leptin concentrations were also lower in DR dams at day 18.5 compared to controls (p<0.05; Figure 2.3D). DR maternal food intake and weight gain during pregnancy were not measured because the aim of the study was to produce IUGR fetuses in response to dietary-restriction and we attempted to minimize other sources of stress, such as handling and restraint.
Figure 2.1: Maternal dietary intake and weight gain. Caloric intake (A) and body weight (B) of CON dams fed *ad libitum* (solid line; n=4-14). A calculated 30% reduction from the average control caloric intake (indicated by the dotted line) was used to determine the calories provided to the dietary-restricted (DR) group. Data are means ± SEM.
Figure 2.2: **Maternal hormonal analysis.** Serum glucose (A), insulin (B), glucose:insulin ratio (C) and leptin (D) concentrations at d18.5 in control (CON) or calorically restricted (DR) dams. Data are means ± SEM, n values on histograms represent whole litters. *p<0.05, ***p<0.001.

### 2.3.2 Fetus

CON and DR pregnant mice produced litters of similar size with similar male:female ratios (Table 2.1). Both male and female fetuses of DR mothers were significantly lighter at day 18.5 than fetuses of CON mothers (p<0.0001, Figure 2.3). There was no impact of sex on fetal body weight (Figure 2.3).
DR male fetuses had a trend towards lower blood glucose compared to CON male fetuses, but this did not reach statistical significance (p=0.06). Serum insulin concentrations and glucose:insulin ratio were not different between male fetuses of CON and DR mothers at day 18.5 (Figure 2.4). Serum glucose and insulin concentrations were not different between female fetuses of CON and DR mothers at day 18.5 (Figure 2.5A,B), although the glucose:insulin ratio was higher in DR female fetuses compared with CON (p<0.05, Figure 2.5C). Further, female fetuses of DR mothers had lower serum leptin concentrations than CON female fetuses (p<0.05; Figure 2.5D). Small sera volumes prevented measurement of male leptin levels.

We have demonstrated that a 30% caloric restriction from mid- to late-gestation produces IUGR offspring, which is consistent with our previous findings [85]. DR decreased maternal glucose, insulin, glucose:insulin ratio, and leptin levels. DR did not appear to affect circulating concentrations of glucose or insulin in male fetuses at day 18.5, however in females, DR was associated with increased glucose:insulin ratio and reduced leptin concentrations.

Table 2.1: Litter size and sex ratio at d18.5 in control (CON) or dietary-restricted (DR) pregnant dams. Data are means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>CON (n = 12)</th>
<th>DR (n = 10)</th>
</tr>
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<tbody>
<tr>
<td>Litter Size</td>
<td>8.54 ± 0.39 (range: 7-11)</td>
<td>8.22 ± 0.55 (range: 6-10)</td>
</tr>
<tr>
<td>Percentage of males (%)</td>
<td>54 ± 4 (range: 25-70)</td>
<td>50 ± 5 (range: 33-78)</td>
</tr>
</tbody>
</table>
Figure 2.3: **Fetal bodyweights.** Bodyweights at d18.5 in male (A) and female (B) fetuses from control (CON) or calorically restricted (DR) dams during pregnancy. Data are means ± SEM, n values on histograms represent whole litters. *p<0.05.
Figure 2.4: **Fetal male hormonal analysis.** Serum glucose (A), insulin (B), and glucose:insulin ratio (C) at d18.5 in control (CON) and dietary-restricted (DR) male fetuses following maternal caloric restriction during pregnancy. Data are means ± SEM. n values on histograms represent whole litters.
Figure 2.5: Fetal female hormonal analysis. Serum glucose (A), insulin (B), glucose:insulin ratio (C), and leptin (D) concentrations at d18.5 in control (CON) and dietary-restricted (DR) female fetuses. Data are calculated as means ± SEM. n values on histograms represent whole litters. *p<0.05.

2.4. Discussion

Male and female fetal bodyweights were decreased in response to dietary-restriction, consistent with the previous findings [85]. Similar to some IUGR rodent models, the caloric restricted diet in the present study did not alter fetal glucose or insulin [120, 202]. This may suggest that
compensatory mechanisms are being induced to adapt to maternal undernutrition. The majority of glucose in the fetus originates from the maternal circulation [203, 204]. Hepatic gluconeogenesis has been shown to be upregulated in fetuses following maternal undernutrition as a means of adapting to low nutrient conditions [131, 205, 206]. Hence in our model fetal hepatic glucose production may be increased to compensate for the lack of maternal nutrition. If this is true, it may be that upregulated hepatic gluconeogenesis may be maintained postnatally, setting the offspring on trajectories to glucose intolerance and metabolic disease [62, 207]. Dietary restriction did not modify male and female insulin concentrations, likely due to the lack of affect on fetal glucose levels. Future studies should investigate glucose and insulin metabolism to elucidate why fetal glucose and insulin levels were not impacted by dietary restriction. Despite the lack of effect of maternal DR on fetal glucose and insulin concentrations, the glucose:insulin ratio was elevated in dietary restricted female fetuses compared with controls. It is unclear why female glucose:insulin ratio are more affected than males.

Reduced glucose, insulin and leptin concentrations in DR dams are likely a result of limited maternal nutrition. Maternal glucose is derived by maternal metabolism from dietary carbohydrates and gluconeogenic amino acids [208]. Complete maternal starvation during pregnancy for 3-4 days significantly lowers maternal blood glucose [209, 210]. These findings correspond with other animal studies of maternal undernutrition that show a positive correlation between maternal caloric intake and plasma glucose concentrations in pregnancy [96, 120]. Insulin is not normally released when circulating glucose levels are low or otherwise a hypoglycemic state would develop [21]. Thus, in the present study, low maternal glucose concentrations may account for reduced insulin levels as a result of antenatal dietary-restriction. Such data are consistent with other maternal undernutrition studies [96]. Leptin is an indicator of positive energy balance and is stimulated by insulin [184, 211]. The reduction in maternal leptin levels seen in our study is consistent with dietary restriction and reduced insulin concentrations. Similar reductions in leptin levels have been reported in dietary-restricted sheep [212] and in human IUGR newborns and their mothers [186, 213]. Leptin crosses the placental barrier [197, 214, 215], thus low maternal leptin levels may help account for the reduced leptin levels in the fetus.
In female fetuses, serum leptin concentrations decreased with dietary restriction. The reduced fetal leptin may, in part, contribute to the observed reduction in fetal body weight since fetal growth is modulated by leptin and leptin receptors are expressed in various fetal mouse tissues, such as cartilage/bone and lung [216, 217]. In human fetuses, growth and development of adipose tissue in late gestation is associated with fetal leptin concentrations [186]. Fetal adipose tissue increases fetal leptin concentrations, and IUGR newborns have reduced leptin concentrations [186]. Maternal undernutrition reduces fetal nutrient supply, energy excess and consequently adipose tissue synthesis [218]. In turn, the reduction in number of adipocytes leads to low leptin secretion, impacting hypothalamic maturation and priming the fetus for impaired appetite regulation and obesity in adulthood [187, 189]. Thus, the reduced fetal female leptin levels with dietary restriction, may result from reduced maternal leptin levels providing less leptin to cross the placenta into fetal circulation [197], and/or low fetal adipocyte leptin secretion as a result of reduced fetal adipocytes and low fetal glucose [187, 218]. We have previously found that in contrast to males, dietary restricted females are resistant to developing some symptoms of the metabolic syndrome [106]. Although we did not measure leptin concentrations in male fetuses, their circulating leptin concentrations may also be reduced following maternal dietary restriction. If this is the case, then both sexes may be programmed for metabolic disease in later life. Females may be less susceptible to developing the metabolic syndrome phenotype due to other factors, such as different sex hormones, or because a secondary challenge is required to induce the metabolic disease phenotype, such as a high-fat diet. If male fetuses do not respond in the same manner as females to maternal undernutrition and their leptin levels do not change, it would be an indication of sex differences in fetal response to maternal undernutrition. Sex differences are important when considering leptin because they are present in early postnatal life, where female newborns have higher cord blood leptin levels than males [219-222], and this trend is maintained into postnatal life where adult women have higher plasma leptin in circulation than men [222]. Dietary restriction lowers fetal glucose levels, which are associated with low leptin levels. DR female fetuses have decreased leptin levels most likely because the maternal source of leptin is low. Furthermore, there is a low level of fetal glucose for adipose tissue maturation, which may lead to a lack of adipocytes to secrete leptin into fetal blood circulation.
In summary, using a similar protocol to that used in the previous animal facility (30% global caloric restriction between days 5.5 and 18.5), we were able to induce a similar level of fetal growth-restriction [85]. In our previous studies, we found that male offspring were more affected by antenatal dietary restriction than female offspring [85], developing obesity, glucose intolerance and insulin resistance in adulthood. Therefore, we chose to utilise male offspring only in the research studies described in this thesis.
Chapter 3
The Influence of Dietary Restriction on Fetal Glucose and Insulin Metabolism

3.1 Introduction

It is now well-demonstrated that intrauterine growth restriction (IUGR) is associated with increased risk for developing insulin resistance in adulthood that leads to the full onset of type 2 diabetes [158, 223-228]. However, the mechanisms by which IUGR leads to these conditions have not been fully defined. IUGR offspring grow rapidly in early postnatal life compared to offspring of normal birthweight and have increased adiposity in childhood and later adult life [229] [158, 230, 231]. Increased adiposity is associated with inflammatory processes capable of stimulating inducing resistance by inhibiting the insulin signalling pathway [21, 232, 233]. Animal models of maternal undernutrition, have identified abnormal pancreatic development in IUGR fetuses that may lead to β-cell dysfunction and the impaired ability to regulate glucose homeostasis in later life [114, 121, 181]. These studies focus on the later development of metabolic disease in IUGR offspring, but the predisposition toward becoming insulin resistant may begin earlier during fetal life.

The insulin signalling pathway is responsible for interpreting the insulin signal into actions, including increasing glucose uptake blood glucose level, inhibition of glucose production and up-regulating glucose storage as glycogen in response to elevated blood glucose levels [20, 21]. Elevated blood glucose levels trigger pancreatic secretion of insulin, which initially binds to and stimulates autophosphorylation of the insulin receptor (INSR) [234] (Figure 3.1). Activation of the insulin receptor’s kinase activity leads to tyrosine phosphorylation of insulin receptor substrates (IRS’s), such as IRS1 and IRS2, which engage downstream signalling molecules to allow the insulin signal transduction to continue [233, 235]. Various combinations of INSR, IRS1 and IRS2 null mutations in mice have revealed their tissue-specific roles in insulin signalling, with IRS1 largely involved in mediating insulin action in skeletal muscle and IRS2 in liver, and ablation of IR increasing insulin resistance in both tissues [236]. IRS1 knockout mice are growth-
restricted at birth, and show a mild state of insulin resistance in adulthood [237, 238], whereas IRS2 knockout mice develop severe insulin resistance in adulthood with inadequate insulin secretion from β-cells to overcome peripheral insulin resistance and eventually die [239].

De novo glucose synthesis, or gluconeogenesis, in the liver is the major source of glucose in the starved state [122], with phosphoenolpyruvate carboxykinase (PEPCK) being one of the major rate-limiting enzymes of the gluconeogenic pathway [132] (Figure 3.1). One of the major pathways through which insulin inhibits hepatic gluconeogenesis is by downregulating PEPCK gene transcription, which decreases hepatic gluconeogenesis [132]. When blood glucose levels are high, such as in the postprandial state, the liver plays a major role in clearing glucose from the circulation. This is apparent by the liver’s capacity to take up one-third of an oral glucose
load [240] and to store it as glycogen to be accessed during fasting periods [29]. Enzymes within the liver that contribute to glycogen metabolism include: glycogen synthase 2 (Gys2), which is involved in glycogen biosynthesis [241]; liver glycogen phosphorylase (Pygl), which catalyzes the degradation of glycogen to glucose-1-phosphate by the phosphorylytic cleavage of 1,4-glycosidic bonds [242]; and phosphorylase kinase (which consists of several subunits, including an alpha subunit, Phka) that stabilizes PYGL in the active conformation [243] (Figure 3.1). The phosphorylation state of these enzymes determines whether they are active and if glycogen will be synthesized or broken down [244]. Increased hepatic glycogen synthesis is a major downstream effect of increased insulin signalling that can be quantified, and thus, hepatic glycogen is often measured in animal studies as a marker of altered glucose metabolism and insulin sensitivity [24, 25].

Previously we have shown that a 30% caloric restriction from mid-late gestation produces IUGR fetuses [85] that develop glucose intolerance and insulin resistance in adulthood [106]. Our animal model presents a unique opportunity to investigate whether there are changes occurring in fetal life that contribute to the development of insulin resistance in adulthood.

We hypothesized that a 30% maternal caloric restriction from mid-late gestation would alter glucose metabolism and insulin signalling in d18.5 fetuses and these changes may influence the onset of metabolic disease in later life. Specifically, we aimed to determine the effect of maternal DR on fetal hepatic glycogen synthesis, a marker for insulin signalling, and on expression of key genes involved in hepatic glucose and glycogen metabolism, in CON and DR fetuses. Further, we aimed to determine the fetal hepatic insulin signalling response to insulin exposure following maternal DR.

### 3.2 Materials and Methods

#### 3.2.1 Animals and Diet

All experiments were approved by the Samuel Lunenfeld Research Institute Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. Inbred C57BL/6J (B6) mice were fed a Dustless Precision Pellets® (Bio-Serv S0173, [http://www.bio-](http://www.bio-))
serv.com/Rodent_DPPS/DPP_RGB.html, Frenchtown, NJ) and sterile water ad libitum under standard conditions as previously described (See Chapter 2). Primiparous females were mated with B6 males and the visualization of a vaginal sperm plug on day 0.5 (d0.5) was indication of successful mating. Between d6.5 and d17.5 of pregnancy, mice were either fed ad libitum (CON) or subjected to a 30% global dietary restriction (DR). On d18.5, a subset of pregnant females was used to evaluate fetal hepatic glycogen synthesis (Study 1). A second cohort of pregnant females was euthanized on d18.5 (see Chapter 2 for details of tissue collection) (Study 2). Fetal heads, livers, placentae and adrenal glands were collected, weighed, flash frozen in liquid nitrogen and stored at -80°C. A third group of pregnant females were weighed from d6.5-d18.5 (CON, n=1; DR, n=9) euthanized by cervical dislocation on d18.5 (Study 3). For this third group of mice, maternal blood was collected in Drummond EDTA-coated microcapillary tubes (VWR, Radnor, PA), allowed to clot on ice in Axygen Scientific 1.5mL eppendorf tubes (VWR, Radnor, PA), centrifuged at 13000 rpm 4°C for 15 min, and the resulting serum was stored at -80°C. Fetuses were extracted, anesthetized on ice, weighed and euthanized by decapitation. Fetal trunk blood glucose was measured using the One Touch Ultra Glucometer (LifeScan Canada Ltd, Burnaby, BC). The remaining fetal trunk blood was collected in Drummond EDTA-coated microcapillary tubes (VWR, Radnor, PA), allowed to clot on ice in Axygen Scientific 1.5mL eppendorf tubes (VWR, Radnor, PA), centrifuged at 13000 rpm 4°C for 15 min, and the resulting serum was stored at -80°C. Maternal and fetal serum insulin and leptin concentrations were measured (see Chapter 2) and fetal liver tissues were collected on ice to evaluate hepatic insulin signalling.

3.2.2 Glycogen Synthesis Rate Evaluation (Study 1)

3.2.2.1 Measurement of fetal hepatic glycogen synthesis

Glycogen synthesis evaluation experiments were adapted from previously published reports [245-247]. In brief, on d18.5, 30 μCi [U-14C]d-glucose (Moravek Biochemicals Inc., Brea, CA, USA) was mixed with 30μl of 10X phosphate buffered saline (PBS) and was injected intraperitoneally into control and DR dams (CON, n=5; DR, n=6). After 6 hours, animals were euthanized by cervical dislocation and fetuses were extracted from the uterine horns. Fetuses were euthanized by decapitation and tail clippings were kept at -20°C for later genotyping.
Placentae were individually removed, weighed and discarded. Fetal livers were individually removed, weighed, rinsed in PBS to remove excess blood and solubilized in 0.6 mL 10N KOH (Fisher Scientific, Nepean, ON, Canada) using a PRO Scientific PRO200 Homogenizer (Diamed, Missisauga, ON, Canada). Tissue lysates were boiled with 8 µg of cold glycogen carrier (Glycogen from oyster, Type II, Sigma-Aldrich, St. Louis, Missouri, USA) for 30 minutes at 100°C. 2 volumes of ethanol were added to the tubes, which were shaken and then kept overnight on ice to allow for glycogen precipitation. Following centrifugation at 16,000 g for 30 min the supernatant was discarded and the glycogen pellets were washed with 66% ethanol and resuspended in 1 mL water. Radioactivity of whole samples was measured as disintegrations per min (dpm) for 10 min using a LS 6500 liquid scintillation counter (Beckman Coulter, Brea, CA, USA).

3.2.2.2 Sex Determination of Fetuses

Genomic DNA was extracted from frozen fetal tails using the REDExtract-N-Amp Tissue PCR kit according to manufacturer’s directions (SIGMA-Aldrich Canada Ltd., Oakville, ON, Canada). In brief, 1 cm of fetal tail was incubated in 125 µL cell lysis solution for 10 min at room temperature. The sample was heated to 95°C for 3 min and 100 µL of neutralizing buffer was added to extract genomic DNA. Fetal sex was determined in a single PCR reaction using a protocol developed by Clapcote and Roder, whereby a set of three primers (Table 1) were used to produce DNA amplicons of different sizes, corresponding to an X (Jarid1c, 301bp) or Y (Jarid1d, 331bp) chromosome-specific gene [248]. These amplicons were visualized under UV light on a 2% agarose gel after electrophoresis at 100 V for approximately 1 h. The presence of two bands indicated a male fetus, whilst the presence of one band indicated a female fetus.

3.2.3 Insulin Signalling and Glucose Metabolism Gene Expression in Fetuses (Study 2)

3.2.3.1 Hepatic mRNA extraction and cDNA synthesis

Total RNA was extracted from frozen embryonic (d18.5) livers (see Chapter 2) using the Allprep DNA/RNA Micro kit (Qiagen, Valencia, CA) (CON, n=6; DR, n=6). In brief, frozen livers (1 mm³) were ground into a powder under liquid nitrogen using a mortar and pestle set on dry ice.
Liver tissue was lysed and homogenized in a guanidine-isothiocyanate–containing buffer. The lysate was then passed through an AllPrep DNA spin column, which bound genomic DNA. The column was washed to remove any contaminants followed by elution of pure DNA. Ethanol was mixed with the flow-through from the AllPrep DNA spin column and then applied to an RNeasy spin column, where total RNA bound to the membrane and contaminants were washed away. RNA was then eluted in 30 µl of water. RNA quality was assessed using the ExperionTM automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA) according to manufacturer’s directions. The Experion system’s software automatically calculates and reports an RNA quality indicator (RQI) value that reflects the integrity of the RNA sample based on several criteria [249]. The RQI values provide an objective assessment of the integrity of the RNA sample and were used to screen out degraded samples [250]. RNA was quantified using the Nanodrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA).

3.2.3.2 Real-Time Polymerase Chain Reaction (RT-PCR)

cDNA was prepared from 1 µg of RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The resulting cDNA was diluted 20-fold, and a 1-µL aliquot was used in a 10- µL PCR reaction (SYBR Green; Sigma, Oakville, ON) containing primers at a concentration of 3 µM each. PCR reactions were run in triplicate and quantitated using the CFX 384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and CFX Manager™ Software (Bio-Rad Laboratories, Hercules, CA). Gene expression was measured for: phosphoenolpyruvate carboxykinase (PEPCK), glycogen synthase 2 (Gys2), glycogen phosphorylase (Pygl), phosphorylase kinase alpha 2 (Phka2), insulin receptor (IR), insulin receptor substrate-1 (IRS1), and insulin receptor substrate-2 (IRS2). The following reference genes were used: beta-actin (Bactin), hypoxanthine guanine phosphoribosyl transferase (Hprt), cyclophilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for Gys2 were obtained from a published report [251]. Primers for PEPCK, Pygl, Phka2, IR, IRS1, IRS2, B-actin, Hprt, Cyclophilin and GAPDH. Primer sequences were obtained from previously published studies or were designed using the Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Table 3.1). Four stable reference genes were selected using the geNorm method [252], which calculated the target stability between the
different conditions (medgen.ugent.be/genorm/). RT-PCR data was calculated for the relative expression ratio of sample versus a control using the equation established by Pfaffl [253]:

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta C_{\text{ref}}(\text{control-sample})}}
\]

The threshold cycle (Ct) is the cycle in which there is the first detectable significant increase in fluorescence; \(E_{\text{target}}\) is the RT-PCR efficiency of the target gene transcript; \(E_{\text{ref}}\) is the RT-PCR efficiency of the reference gene transcript; \(\Delta C_{\text{target}}\) is the difference in target gene mean expression levels between DR and CON males; \(\Delta C_{\text{ref}}\) is the difference in average reference gene mean expression levels between DR and CON males.

Table 3.1: Sequences of the primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Source (citation or NCBI accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phka2</td>
<td>GGAGAGAAAGCGTGCGCCCAA</td>
<td>TCTGGTGATGCCACTCCGCTCA</td>
<td>NM_172783.3</td>
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<tr>
<td>PYGL</td>
<td>CTGGCTGACTTGATAGCGGAGAAA</td>
<td>CCCGGAGGAAGATGTCATCAGCTCA</td>
<td>NM_133198.2</td>
</tr>
<tr>
<td>GYS2</td>
<td>GGACTGGGCTGATCCTTTTCTC</td>
<td>GCAGTGGCGATGGTTGTA</td>
<td>Liu, 2007 #541</td>
</tr>
</tbody>
</table>
3.2.4 Fetal Hepatic Insulin Challenge (Study 3)

The fetal livers were dissected free and pooled together within a litter. Total liver tissue was rinsed with a 0.9% sodium chloride irrigation solution (Baxter Corporation, Toronto, ON, Canada) to remove excess blood and minced on ice. Minced liver homogenate was aliquoted into 7 equal aliquots of approximately 30 mg samples of pooled liver tissue per tube. 217 µL of HBSS with Ca²⁺/Mg²⁺ (Biowhitaker, Walkersville, MD), 25 mM HEPES (Biowhitaker, Walkersville, MD), pH = 7.4 (HBSS++) was added to each tube. The tissue was treated with a final concentration of insulin of 100 nM. An insulin concentration of 100 nM was selected based on previous reports showing stimulation of insulin signalling and glycogen synthesis with administration of 100nM insulin to 6.8 x 10⁴ cells/cm² [254] or 0.5 x 10⁶ cells [245]. Samples were incubated in a shaking waterbath at 37°C (SW22, Julabo, Allentown, PA, USA) for 0, 1, 5, 15, 30, 60, or 180 min. A negative control tube with no insulin addition was taken at 60 min and at 1, 5, and 15 min when there were sufficient amounts of tissue. After each timepoint, samples were placed on ice and washed with Cell Wash buffer (Bio-Plex Signal Transduction Reagents
and Cell Lysis Kit, Bio-Rad Laboratories Ltd., Hercules, CA). Samples were then centrifuged briefly to pellet the tissue, and the supernatant was aspirated and discarded. 225 μL of cell lysis buffer (Bio-Plex Signal Transduction Reagents and Cell Lysis Kit, Bio-Rad Laboratories Ltd., Hercules, CA) was added to the pellet, which was then homogenized for 30 sec (PRO Scientific PRO200 Homogenizer, Diamed, Missisauga, ON, Canada). The samples were allowed to sit on ice for 15 min and then spun at 14000 rpm at 4°C for 15-20 min. The supernatant containing protein was transferred to a new tube. Protein concentrations in the supernatant were measured using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, Il).

3.2.4.1 Western Blotting And ELISAs

These experiments utilised enzyme linked immunosorbent assays (ELISA) to quantify phosphorylated- and total protein levels because of ELISA’s high sensitivity and specificity in comparison to western blots [255]. We first performed preliminary testing using western blotting to measure phospho-glycogen synthase kinase 3 (P-GSK3) and phospho-insulin receptor substrate 1 (IRS1) to determine the effect of insulin treatment on fetal hepatic insulin signalling in vitro (CON, n=1). These initial western blots served as a cost-effective way to confirm that the insulin-challenge protocol was stimulating insulin signalling (data not shown).

30 µg liver protein samples (CON, n=5; DR, n=7) were used in each ELISA for quantification of Insulin receptor (IR) (Millipore, Billerica, MA), GSK3 (R&D Systems, Minneapolis, MN) and Phospho-GSK3 (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. Data was calculated as protein expression level divided by amount of protein loaded per ELISA and then normalized to time = 0 min (baseline).

3.2.5 Statistical Analysis

Data are presented as means ± standard errors of the mean (SEM). Number of litters (biological replicates) is indicated on the histograms. Statistical analyses were performed using JMP 9.0.2 (SAS Institute Inc, Cary, NC). Data were assessed for normality using the Kolmogorov-Smirnov test. Data were analyzed by Student’s t test with maternal diet as the factor. The influence of sex differences on the data was determined by two-way ANOVA, followed by post-hoc Tukey
univariate tests, with maternal diet and sex as the factors. Data that was not and could not be transformed to be normally distributed were analyzed by Wilcoxon test. The repeated measures one-way ANOVA, followed by post-hoc Tukey univariate tests, was used to evaluate the change in maternal weight change from baseline over time with diet and time as the factors, and protein concentrations over time with insulin treatment and time as the factors. P values less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Growth Restriction and Decreased Glycogen Synthesis Rate (Study 1)

Dietary restricted female and male fetuses had lower body weights (p<0.0001, Table 3.2) and increased placental:body weight ratios than control females and males, respectively (p<0.05, Table 3.2). CON male fetuses were heavier than CON female fetuses (p<0.05, Table 3.2). 6 hours after the [U-14C]D-glucose injection, both dietary restricted male and female offspring synthesized less glycogen compared to fetuses from control dams (p<0.05, Figure 3.2).
Table 3.2: **Body weights and placental weight:bodyweight ratios in fetuses** from control (CON) or dietary-restricted (DR) pregnant dams at d18.5 used for liver analysis. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.0001, significantly different from same-sex CON.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON (n = 5)</td>
<td>DR (n = 6)</td>
</tr>
<tr>
<td></td>
<td>CON (n = 5)</td>
<td>DR (n = 6)</td>
</tr>
<tr>
<td>Body weights (g)</td>
<td>1.24 ± 0.02</td>
<td>0.97 ± 0.02***</td>
</tr>
<tr>
<td></td>
<td>1.19 ± 0.01</td>
<td>0.97 ± 0.01***</td>
</tr>
<tr>
<td>Placental/Bodyweight (x10^-1 g/g)</td>
<td>0.69 ± 0.04</td>
<td>0.84 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>0.65 ± 0.02</td>
<td>0.77 ± 0.03**</td>
</tr>
</tbody>
</table>

Figure 3.2: **Rate of fetal hepatic glycogen synthesis.** Incorporation of [U-^{14}C]D-glucose into the hepatic glycogen of male (A) and female (B) fetuses from control (CON) and dietary-restricted (DR) dams at 6-hours post-intraperitoneal exposure on d18.5. Data are means ± SEM. *p<0.05, ***p<0.0001.
3.3.2 Altered Hepatic Gene Expression (Study 2)

3.3.2.1 Glucose and Glycogen Metabolism

Fetal male blood glucose and insulin levels were not affected by maternal diet (Chapter 2, Figure 2.4). Dietary-restricted males had significantly higher hepatic gene expression of *Phka2* (p<0.05) and trended towards having increased *PEPCK* expression (Figure 3.3). There was no effect of maternal diet on hepatic *Pygl* and *Gys2* mRNA expression (Figure 3.3).

![Fold change in hepatic gene expression](image)

Figure 3.3: **Fold change in hepatic gene expression of glucose/glycogen metabolism** in dietary restricted (DR) male fetuses relative to expression in control (CON) male fetuses (represented by the horizontal line). Bars represent 95% confidence limits and may lie within the symbol. Confidence limits that do not cross one are significantly different from CON at p<0.05.

3.3.2.2 Insulin Signalling

Dietary-restricted fetal males had significantly higher gene expression of *IRS1* (13%) and *IRS2* (19%) than males from control-fed dams at d18.5 (p<0.05, Figure 3.4). There was no significant effect of maternal diet on hepatic *IR* mRNA expression (Figure 3.4).
Figure 3.4: **Fold change in hepatic gene expression of insulin signalling genes** in dietary restricted (DR) male fetuses relative to expression in control (CON) male fetuses (represented by the horizontal line). The 95% confidence limits lie within the symbols. Confidence limits that do not cross one are significantly different from CON at p<0.05.

### 3.3.3 Hepatic Insulin Signalling Challenge (Study 3)

#### 3.3.3.1 Mothers

Dietary restricted dams weighed less than control dams fed *ad libitum* at d18.5 gestation (p<0.05, Figure 3.5). DR dams also had significantly lower blood glucose (p<0.0001, Figure 3.6A), serum insulin (p<0.0001, Figure 3.6B) and serum leptin (p<0.01, Figure 3.6D) levels compared to CON. There was no difference in glucose:insulin ratios (Figure 3.6C) between CON and DR dams.
Figure 3.5: **Maternal weight gain (Study 3).** Maternal weight change relative to baseline weight before mating from d6.5 to d18.5 of pregnancy. Data are means ± SEM.
Figure 3.6: **Maternal hormonal analysis (Study 3)**. Blood glucose (A), serum insulin (B), glucose:insulin ratio (C), and serum leptin (D) levels in CON and DR mothers at d18.5.

**p < 0.01, ***p < 0.0001.

### 3.3.3.2 Fetuses

In response to maternal caloric restriction, both male and female fetuses had reduced body weight at d18.5 (p < 0.0001, Table 3.3). Irrespective of maternal diet, sex had a significant impact on fetal body weight (p < 0.05, Table 3.3). Maternal dietary restriction lowered both male and female fetal blood glucose (p < 0.0001), serum insulin (p < 0.05) and serum leptin levels (p < 0.05) compared to control fetuses (Figure 3.7A,B,D, Figure 3.8A,B,D). Compared to males from control dams, dietary restricted males had lower insulin (Figure 3.7B) and similar glucose:insulin ratios (Figure 3.7C). Maternal dietary restriction did not alter fetal glucose:insulin ratios (Figure
3.7C, Figure 3.8C). In dietary restricted litters, male fetuses had lower blood glucose concentrations than female fetuses (p<0.05, Figure 3.7A, Figure 3.8A).

Table 3.3: **Fetal bodyweights (Study 3).** Body weights in control (CON) and dietary-restricted (DR) fetuses at d18.5. Data are means ± SEM. ***p<0.0001, significantly different from same-sex CON.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td></td>
<td>CON (n=7)</td>
<td>DR (n=9)</td>
</tr>
<tr>
<td>Body weights</td>
<td>1.24 ± 0.02</td>
<td>0.99 ± 0.02***</td>
</tr>
<tr>
<td>(g)</td>
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</table>
Figure 3.7: **Fetal male hormone analysis (Study 3).** Blood glucose (A), serum insulin (B), glucose:insulin ratio (C) and serum leptin (D) levels in male fetuses at d18.5 from control (CON) and dietary-restricted (DR). Data are means ± SEM, n values on histograms represent whole litters. *p<0.05, ***p<0.0001.
3.3.3.3 Hepatic Insulin Signalling

Following insulin challenge, dietary restriction reduced fetal hepatic IR protein expression over time (p<0.01) but there was no difference in IR protein expression between the dietary groups over time (Figure 3.9A). There was neither an effect of time nor maternal diet on total IR protein expression in negative controls, although total protein levels at each time point were similar to levels in stimulated samples at the same corresponding time point (Figure 3.9B).
There was neither an effect of time nor maternal diet on Phospho-GSK3:Total GSK3 protein expression (Figure 3.10A). Although negative controls were not different between dietary groups, there was an effect of time on Phospho-GSK3:GSK3 protein expression in negative controls (p<0.05), and total protein levels at each time point were similar to levels in stimulated samples at the same corresponding time point (Figure 3.10B).
Figure 3.9: **Total insulin receptor (IR) protein concentration.** IR protein concentration in pooled fetal livers after insulin exposure from 0 to 180 minutes ($A$) or in the absence of insulin ($B$) in control (CON) and dietary restricted (DR) dams at d18.5. Data are means ± SEM. There were no negative controls (absence of insulin) for time points 30 and 180.
Figure 3.10: **Phospho-GSK3:GSK3 protein concentration ratio.** Phospho-GSK3:GSK3 protein ratio in pooled fetal livers after insulin exposure from 0 to 180 minutes (A) or in the absence of insulin (B) in control (CON) and dietary restricted (DR) dams at d18.5. Data are means ± SEM. There were no negative controls (absence of insulin) for time points 30 and 180.
3.4 Discussion

These data support the hypothesis that maternal dietary restriction can impact fetal growth and influence fetal glucose metabolic function. This may have potential long-term effects on hepatic metabolic function, which may contribute to the increased incidence of glucose intolerance, insulin resistance and obesity observed in dietary restricted adult male offspring [106].

Maternal dietary restriction alters maternal and fetal weights, and leads to changes in hepatic glucose metabolic function in term fetuses. Both male and female DR fetuses demonstrated a decreased rate of hepatic glycogen synthesis compared to control fetuses. There have been several animal studies of maternal undernutrition that measured overall hepatic glycogen content in fetuses with differing results. In sheep, placental insufficiency produces growth-restricted fetuses with lower glucose and insulin levels, but similar overall hepatic glycogen content compared to control fetuses [206]. Similarly, maternal low protein diet throughout gestation did not affect glucose and insulin levels, or liver glycogen content in growth-restricted rat fetuses [202]. Alternatively, a rat model of uterine artery ligation in late gestation used histological methods to show a decrease in liver glycogen in growth-restricted fetuses [256]. Maternal caloric restriction in late gestation also reduced hepatic glycogen content in fetal mice [257]. These studies taken together suggest that maternal undernutrition differs in its impact on fetal liver glycogen metabolism when implemented during different periods of gestation and in different animal species.

One possible explanation for reduced fetal hepatic glycogen production in DR fetuses is a decrease in glycogen synthesis. DR fetuses had lower glucose and insulin concentrations compared to controls. Glucose and insulin are major driving forces of glycogen synthesis, with glucose as the major substrate and insulin stimulating the pathways of glycogen production [19, 22]. Low glucose concentrations in DR fetuses may be a reflection of low maternal glucose concentrations in these dams since fetal glucose originates from the maternal circulation throughout gestation [203, 204, 258]. By late gestation, fetal insulin is glucose-regulated and helps maintain blood glucose homeostasis [259]. Thus, reduced fetal glucose levels would account for the decrease in fetal insulin levels in dietary restricted fetuses. The absence of insulin
may then explain a decrease in glycogen synthesis and removal of inhibition on gluconeogenesis [21]. Maternal dietary restriction lowers fetal glucose and insulin levels, which may account for the decreased rate of glycogen synthesis in fetal livers.

Alternatively, there may be abnormalities in expression of genes that are involved in glycogen metabolism. Hence, we measured gene expression of enzymes that mediate hepatic glucose metabolism and insulin signalling. If mRNA expression is a reflection of protein expression and enzymatic activity, then the slight elevation in glycogenolytic Phka2 mRNA expression may indicate increased glycogen breakdown in DR fetuses compared to controls, while the lack of change in Gys2 and Pygl gene transcription may indicate no difference in glycogen synthesis. Moreover, elevation in Phka2 in dietary restricted fetuses may suggest increased phosphorylation, and thus activation, of Pygl leading to an increased rate of glucose release from stored glycogen in DR fetuses compared to controls [244]. Further, a lack of alteration in fetal Gys2 enzymatic activity with maternal undernutrition may indicate similar glycogen synthesis rates between dietary restricted and control fetuses [244]. A study by Hsu et al. (1993) has also demonstrated that hepatic glycogen synthase activity does not change with maternal caloric restriction in late gestation fetal mice [257]. Dietary restricted fetuses had altered gene expression of enzymes involved in glycogen metabolism, but they were not marked changes. There were slight but significant changes in hepatic mRNA expression of insulin signalling genes in DR males compared to males from control-fed dams. Maternal dietary restriction elevates hepatic mRNA expression levels of IRS1 and IRS2 in male DR fetuses compared to controls. Over-expression and up-regulation of IRS1 and IRS2 is associated with increased insulin sensitivity in cell lines and mice [234, 260]. In our study, there is a 40% elevation in the hepatic mRNA expression of the gluconeogenic enzyme PEPCK in male fetuses from dietary restricted dams compared to those from control dams. This observation correlates with our previous findings of increased mRNA and protein expression of PEPCK and several other gluconeogenic enzymes [261]. Without the inhibitory action of insulin, the growth-restricted fetus from undernourished dams may try to compensate for low nutrient levels by increasing hepatic gluconeogenic enzyme expression and thus, glucose production. Maternal fasting in rats during late gestation reduces fetal insulin and increases PEPCK activity near term [131]. If
maintained postnatally, the drive towards increased glucose synthesis, as an adaptation to malnutrition, may permanently affect glucose homeostasis and predispose offspring to developing hyperglycemia and potentially glucose intolerance and insulin resistance in adulthood [106]. Other rodent models support this theory and show that maternal undernutrition leads to higher PEPCK enzymatic activity [133], increased hepatic PEPCK mRNA expression [132, 159] and elevated glucose concentrations [132] in adult male offspring compared to controls.

An *in vitro* challenge of fetal livers with insulin showed no evidence of dietary restriction altering the insulin signalling pathway. We measured IR and GSK3 protein expression, which lie at the beginning and end of the insulin signalling pathway, respectively. There was no effect of insulin stimulation on hepatic IR or phospo-GSK3:GSK3 protein expression, in either litter groups, control or dietary restricted. This is surprising considering that the preliminary immunoblots showed an increase in phospo-GSK3 after insulin exposure, and the ELISA results indicates that the protocol requires further optimization. Therefore at this time, we are unable to offer definitive conclusions on the effect of maternal DR on activation of the insulin signalling pathway and whether alterations in this pathway underlie changes in fetal life leading to the development of insulin resistance symptoms seen in adulthood [106].

Leptin is an indicator of positive energy balance and is stimulated by insulin [184, 211]. In response to DR, leptin concentrations were reduced in both male and female fetuses (Study 3), which is consistent with previous observations (Chapter 2, Figure 5). Leptin is secreted by adipocytes and is lower in growth-restricted newborns that are exposed to maternal undernutrition compared to babies of normal birthweight [186, 218]. However, it is important to note that fat mass in the rodent at birth is normally low, and thus a change in fetal fat mass may not account for the change in fetal leptin levels with dietary restriction. There are two possible reasons that are not mutually exclusive for reduced fetal leptin levels with dietary restriction in our model. First, reduced maternal leptin levels may provide less leptin to cross the placenta and enter into fetal circulation [197]. Second, the rodent placenta expresses high levels of leptin and its receptor, which is involved in leptin transport to fetal circulation and shows that the placenta
has a role in regulating fetal leptin levels [216]. Placental size decreases with reduced maternal nutrition and its physiology may also change leading to reduced leptin delivery to fetuses.

Maternal caloric restriction from mid-late gestation reduced the rate of glucose incorporation into hepatic glycogen and lowered glucose and insulin levels in fetuses. There are several possible mechanisms that may account for the decrease in rate of hepatic glycogen synthesis, however our data does not support any one mechanism in particular. There may be changes in fetal glucose metabolism and insulin signalling but the extent to which they predispose the dietary-restricted offspring toward insulin resistance in later life is unclear.
Chapter 4

Omega-3 Fatty Acids Prevent the Development of Obesity, Insulin Resistance and Glucose Intolerance in Male C57BL/6J Mice Offspring Subjected to Maternal Undernutrition

4.1. Introduction

Maternal undernutrition during pregnancy is strongly associated with development of the metabolic syndrome, which is a series of related risk factors associated with type 2 diabetes and cardiovascular disease (including hypertension, hyperglycaemia, insulin resistance and central obesity) in offspring in later life [61, 62, 66-68, 156, 157]. Animal studies have also shown the relationship between poor maternal nutrition and metabolic change in offspring, however, the specific disease phenotype is dependent on the dietary challenge and the timing of that challenge during critical windows of offspring development [61, 105, 113, 201, 262, 263]. While the precise mechanisms by which the developmental programming of adult disease is induced have yet to be fully determined, the impact on the health of individuals is clear and has spurred research into interventions to prevent or mitigate the detrimental effects of adverse early life exposures.

Considerable evidence has emerged revealing the wide-range of health benefits of increasing dietary intake of omega-3 fatty acids (ω-3 FA). It began when researchers realized a lower incidence of coronary heart disease in Greenland Inuit populations compared to Scandinavian controls [264, 265]. The difference in disease risk was attributed to the Inuit diet, which contained an extremely high fish content [266]. Since then, clinical studies have found that ω-3 FA dietary supplements may reduce the metabolic syndrome risk in men [160], and improve diabetes and cardiovascular risk factors in overweight, hypertensive, hyperinsulinemic patients [161-163]. Dietary supplementation with ω-3 FA in rats prevented the onset of hyperleptinemia and hypertension in adulthood following prenatal exposure to dexamethasone [267]. Additionally, rodent models have extensively used dietary ω-3 FA to treat and/or prevent the development of metabolic disease induced by high-sucrose or high-fat diets, and have
demonstrated improvements in hyperinsulinemia, insulin resistance and obesity [268-270]. These findings support the use of dietary ω-3 FA as a nutritional intervention for cardiometabolic disease. 

Glucose tolerance testing and homeostasis model assessment of insulin resistance (HOMA-IR) are appropriate measurements of insulin response and glucose tolerance in both human and rodents [271]. In non-diabetic mice, during glucose tolerance testing, insulin levels normally peak at approximately 15 min post-glucose load, at which time insulin-sensitive tissues begin removing glucose from the blood circulation [272]. Thus, following the 15 min time-point, glucose levels represent insulin sensitivity. HOMA-IR is a rough surrogate index of insulin sensitivity in place of an euglycemic-clamp in mice, and is preferred to the latter because it minimizes animal stress, time and cost [271]. An adjusted HOMA-IR has been established for rodents that more closely correlates with euglycemic-clamp findings [273, 274].

We have previously reported that pregnant C57BL/6J mice fed a 30% caloric-restricted diet during mid- to late-gestation, produce intrauterine growth-restricted offspring with increased placental weights [85]. Male, but not female, offspring of these malnourished mothers later develop indications of glucose intolerance, insulin resistance and obesity in adulthood [106]. In the present study we hypothesize that a diet rich in ω-3 FA would attenuate the development of obesity, insulin resistance and glucose intolerance in adult male offspring subjected to antenatal dietary restriction DR.

4.2. Materials and Methods

4.2.1. Animals and Diet

All experiments were approved by the Samuel Lunenfeld Research Institute Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. Inbred C57BL/6J (B6) mice were fed a standard control diet (1% ω-3 FA; Dustless Precision Pellets® Bio-Serv S0173 http://www.bio-serv.com/Rodent_DPPS/DPP_RGB.html, Frenchtown, NJ) and sterile water ad libitum under standard conditions as previously described (see Chapter 2).
4.2.2. Male Dietary Intake Pilot Study

Adult B6 males, at 56 days of age, were assigned to one of two groups: standard control diet (CON, n=4), or a diet enriched in omega-3 FA (ω-3, n=3)(35% ω-3 FA; Bio-Serv S5606) in which the source of animal fats was replaced by fish oil (Table 1). Before being fed the differing diets and 37 days later, the fat:lean mass ratio and body weights were quantified using dual-energy X-ray absorptiometry (DEXA; PIXI-mus, LUNAR, Madison, WI). In brief, PIXImus2 software was used to calculate body composition of males anaesthetized with isoflurane as previously described [106].
Table 4.1: Composition and fatty acid profile of control and ω-3 diet.

<table>
<thead>
<tr>
<th>Composition (kcal/g)</th>
<th>Control</th>
<th>ω-3</th>
<th>Composition (kcal/g)</th>
<th>Control</th>
<th>ω-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.844</td>
<td>0.844</td>
<td>C14:0 Myristic</td>
<td>0</td>
<td>3.75</td>
</tr>
<tr>
<td>Fat</td>
<td>0.360</td>
<td>0.450</td>
<td>C16:0 Palmitic</td>
<td>0</td>
<td>8.80</td>
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<tr>
<td>Carbohydrate</td>
<td>2.200</td>
<td>2.271</td>
<td>C16:1 Palmitoleic</td>
<td>0</td>
<td>5.75</td>
</tr>
<tr>
<td>Total</td>
<td>3.404</td>
<td>3.565</td>
<td>C18:0 Stearic</td>
<td>0</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C18:1 Oleic</td>
<td>0</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C18:2 Linoleic</td>
<td>12.52</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C18:3 Linolenic*</td>
<td>1.00</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C18:4 Octadecatetraenoic</td>
<td>0</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C20:1 Gadoleic</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C20:4 Arachidonic</td>
<td>0</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C20:5 Eicosapentaenoic*</td>
<td>0</td>
<td>6.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C22:1 Erucic</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C22:6 Docosahexaenoic*</td>
<td>0</td>
<td>6.00</td>
</tr>
</tbody>
</table>

*an ω-3 FA

4.2.3. Maternal Dietary Protocol

Primiparous B6 females were mated with B6 males where the visualization of a vaginal sperm plug on day 0.5 (d0.5) was indication of successful mating. Between d6.5 and 17.5 of pregnancy, females were assigned to one of two groups: fed ad libitum as controls (C), or subjected to antenatal dietary restriction (R) by a 30% global caloric restriction of the diet. After d17.5 of
pregnancy, all mothers were fed ad libitum. The dietary restriction protocol for the R cohort was calculated for each day of pregnancy as previously described [85]. After weaning at 21.5 days postpartum, male pups were randomly assigned to either the control diet (C) or the ω-3 diet (35% ω-3 FA; Bio-Serv S5606) for the remainder of the study. (Table 4.1) resulting in four experimental groups: antenatal control + postweaning control diets (C/C; n=4 offspring); antenatal control + postweaning ω-3 diets (C/ω-3; n=4 offspring); antenatal restricted + postweaning control diets (R/C; n=4 offspring); and antenatal restricted + postweaning ω-3 diets (R/ω-3; n=3 offspring) (Figure 4.1). For this study, 39 male B6 pups from 15 litters were used and n number represents litters.

Figure 4.1: Experimental design for postweaning nutritional intervention following maternal dietary restriction. B6 mothers are fed ad libitum (Controls, C) or a 30% caloric restriction from day 6.5 to 17.5 of gestation (Restricted, R). The male offspring are fed either the control diet (C/C, R/C) or the ω-3 FA enriched diet (C/ω3, R/ω3) starting at 21.5 days of age for the remainder of the study. At 3 and 12 months of age, the offspring are subjected to glucose tolerance testing (GTT). At 12 months of age, body composition is measured using dual-energy x-ray absorptiometry (DEXA).
4.2.4. **Body Composition**

At 12 months of age, the percentage of body fat, lean muscle mass and the area of bone mass were quantified using dual-energy X-ray absorptiometry (DEXA; PIXI-mus, LUNAR, Madison, WI). DEXA was performed as previously described [106]. Central obesity was calculated as epididymal fat pad weight divided by total body weight.

4.2.5. **Glucose Tolerance Test (GTT)**

GTT was conducted at 3 and 12 months of age as previously described [85]. Briefly, animals were fasted overnight with free access to water. Blood was collected from the saphenous vein and again at 30 and 60 min following an intraperitoneal glucose injection (1.5 mg glucose/g body weight). Blood glucose was measured using a One Touch Ultra Glucometer (LifeScan Canada Ltd, Burnaby, BC). Blood was also collected using EDTA-coated Microcap® microcapillary tubes (Drummond Scientific Co, Broomall, PA), stored at 4°C for 2 hours then centrifuged at 4°C and 13000 rpm for 15 min. Serum was extracted and stored at -20°C until needed. Serum Insulin was measured using Insulin (Mouse) Ultrasensitive EIA kits (ALPCO Diagnostics, Salem, NH). There was a <10% interassay and intraassay coefficient of variation for all assays. There was a sensitivity of 0.115 ng/mL for insulin. At 3 and 12 months of age, fasting glucose and insulin levels in blood circulation were used to calculate HOMA-IR. This index was developed using human data but subsequently has been validated in rodents [271, 273, 274].

\[
\text{HOMA-IR} = \frac{\text{Fasting glucose} \times \text{Fasting Insulin}}{2390} \quad \text{(with glucose expressed as mmol/L and insulin expressed as µU/mL)} [273].
\]

4.2.6. **Statistical Analysis**

Data were analyzed by SAS statistical software (Version 9.1, Cary, NC). All males within a dietary group were used for statistical analysis and were not separated based on litter. Data were assessed for normality using the Kolmogorov-Smirnov test. The repeated measures ANOVA was used to evaluate the change in male dietary intake over time with diet and time as the factors. For glucose tolerance, incremental area under the curve (AUC) was first calculated for each individual offspring. To determine the effects of antenatal diet and postweaning and compare
differences among group means, data were analyzed by two-way ANOVA followed by Tukey’s post hoc test. Welch’s or Wilcoxon tests were used for analysis when data was not normally distributed with unequal variances. Data are presented as means ± standard error mean unless otherwise stated. p<0.05 was considered to be statistically significant.

4.3. Results

4.3.1. Male Dietary Intake Pilot Study

Over the 37-day period, adult males fed the control and ω-3 diets had similar daily caloric intake (Figure 4.2). Furthermore, the ω-3 diet did not significantly alter body weight nor fat:lean ratio compared to males fed the control diet (Figure 4.3A,B).
Figure 4.2: **Daily kilocaloric consumption in the dietary intake pilot study.** Caloric intake of males fed the control diet (CON, black line; n=4) or the ω-3 FA enriched diet (ω-3; grey line; n=3) for 37 days. Data are means ± SEM.

Figure 4.3: **Adult male body composition in the dietary intake pilot study.** Body weight (A) and Fat:Lean ratio (B) in males fed the control diet (CON, black line; n=4) or the ω-3 FA enriched diet (ω-3; grey line; n=3) for 37 days. Data are means ± SEM. Histograms with different letters are significantly different (p<0.05).

### 4.3.2. Growth Trajectory and Body Composition of Adult Male Offspring

Bodyweight varied significantly with postweaning diet (p<0.0001) but not with antenatal diet, and there was no significant interaction of postweaning diet with antenatal diet. Antenatal dietary-restriction did not affect bodyweights in males compared to males from control-fed dams at 3 or 12 months of age (Figure 4.4). The postweaning ω-3 diet lowered bodyweight in R offspring at both 3 and 12 months (3 months, R/ω3 vs. R/C: 28% lower, p<0.0001, Figure 4.4A; 12 months, R/ω3 vs. R/C: 14% lower, p<0.0001, Figure 4.4B). The postweaning ω-3 diet also lowered bodyweight in offspring from control dams at both 3 and 12 months (3 months, C/ω3 vs.
Percentage of body fat varied with postweaning diet (p<0.01) but not maternal diet, and there was a significant interaction between antenatal and postweaning diets (p<0.01). Fat:lean ratio varied with postweaning diet (p<0.01) but not maternal diet, and there was a significant interaction between antenatal and postweaning diets (p<0.01). Antenatal dietary-restriction increased obesity and fat:lean ratio in offspring fed the control postweaning diet (obesity, R/C vs. C/C: 31% higher, p<0.05, Figure 4.5A; fat:lean ratio: R/C vs. C/C: 43% higher, p<0.05, Figure 4.5B). Increasing dietary ω-3 FAs lowered obesity and fat:lean ratio of restricted offspring (obesity, R/ω3 vs. R/C: 37% lower, p<0.01, Figure 4.5A; fat:lean ratio R/ω3 vs. R/C: 45% lower, p<0.01, Figure 4.5B). In contrast, the postweaning ω-3 diet did not alter obesity or fat:lean ratio in antenatal control offspring (Figure 4.5A,B).

Figure 4.4: **Body weight measurements in adult offspring at 3 and 12 months.** Body weights at 3 (A) and 12 months (B) of age in offspring of mothers fed control (C) or restricted (R) diet during pregnancy and fed a control or omega-3 (ω-3) diet from weaning. Data are means ± SEM, n values on histograms represent the numbers of males and of whole litters. Histograms with different letters are significantly different (p<0.05).
Figure 4.5: **Fat mass and fat:lean ratio in 12 month old offspring.** Percentage of body fat (A) and total lean mass (B) at 12 months of age in offspring of mothers fed control (C) or restricted (R) diet during pregnancy and fed a control or omega-3 (ω-3) diet from weaning. Data are means ± SEM, n values on histograms represent the numbers of males and of whole litters. Histograms with different letters are significantly different (p<0.05).

4.3.3. **Glucose tolerance test and HOMA-IR**

Figure 4.6A illustrates blood glucose at fasting levels, and at 30 and 60 min following an intraperitoneal glucose load at 3 months of age. Fasting glucose varied with postweaning diet (p<0.0001) but not maternal diet (p=0.06), and there was no significant interaction between antenatal and postweaning diets. Fasting glucose was unaffected by dietary-restriction. However, the postweaning ω-3 diet lowered fasting glucose for antenatal dietary-restricted and antenatal control offspring (R/ω3 vs R/C: 37% lower, p<0.0001; C/ω3 vs C/C: 39% lower, p<0.001). There was no interaction between antenatal and postweaning diets for offsprings’ glucose change from 0 to 30 min; antenatal diet significantly contributed to the results (p<0.01), whilst postweaning diet did not. Dietary-restriction raised the incremental peak in glucose concentrations at 30 min post-glucose load (R/C: 9.65 ± 1.20 mmol/L; C/C: 6.29 ± 1.37 mmol/L, p<0.05). A postweaning ω-3 diet reduced the glucose peak of restricted offspring compared to those fed the standard diet, but the trend was not significant (R/ω3: 6.92 ± 0.55 mmol/L,
Increasing dietary ω-3 FAs did not affect the 30 min glucose peak in offspring from control dams compared to antenatal controls fed the standard diet.

Figure 4.6B shows incremental glucose AUC at 3 months of age. Glucose AUC varied with antenatal diet (p<0.001), but not with postweaning (p=0.0572) diet, and there was no interaction between the diets. Glucose AUC was higher with dietary restriction in offspring fed the standard diet compared to antenatal controls (R/C vs C/C: 80% higher, p<0.05). The postweaning ω-3 diet lowered glucose AUC for restricted offspring compared to those fed the standard diet, but the trend failed to reach statistical significance (R/C vs R/ω3: 28% lower, p=0.0588), and it did not change glucose AUC in offspring from control dams.

Figure 4.6C depicts serum insulin at fasting levels, and at 30 and 60 min following an intraperitoneal glucose load at 3 months of age. Fasting insulin did not vary with antenatal diet or postweaning diet and there was no interaction between them. Insulin incremental peak at 30 min was not associated with maternal diet (p=0.0575) nor postweaning diet and there was no interaction between the two diets. Dietary restriction lead to a higher peak insulin response compared to offspring from control dams when both groups were fed the postweaning standard diet (R/C: 0.253 ± 0.058 ng/mL; C/C: 0 ± 0.021 ng/mL; p<0.01). A postweaning ω-3 diet reduced the insulin peak of restricted offspring (R/ω3: 0.036 ± 0.034 ng/mL; p<0.05), although it had no effect on antenatal control offspring.

Figure 4.6D shows incremental serum insulin AUC at 3 months of age. Insulin AUC varied with antenatal diet (p<0.05), but not with postweaning diet, and there was no interaction between the diets. Dietary restriction lead to significantly higher AUC in offspring fed the standard diet in comparison to those from antenatal control dams (R/C vs C/C: p<0.01). The postweaning ω-3 diet lowered insulin AUC of restricted offspring (R/ω3 vs R/C: p<0.05), but did not change that of mice from control dams.

Figure 4.7A shows blood glucose at fasting levels, and at 30 and 60 min following an intraperitoneal glucose load at 12 months of age. Fasting glucose does not significantly correlate with antenatal diet and postweaning diet, nor was there any interaction between the diets.
Glucose peak response at 30 min varied with antenatal diet (p<0.05) and postnatal diet (p<0.05), but there was no interaction between the two diets. There was no difference in incremental peak glucose at 30 min between groups.

Figure 4.7B shows glucose AUC at 12 months of age. Glucose AUC varied with postweaning diet (p<0.01) but not with antenatal diet, and there was no interaction between the diets. In offspring fed the control diet, dietary-restriction increased glucose AUC compared to antenatal control offspring but not significantly. The postweaning ω-3 diet trended towards reducing glucose AUC in dietary-restricted males, but it was not significant.

Figure 4.7C depicts serum insulin at fasting levels, and at 30 and 60 min following an intraperitoneal glucose load at 12 months of age. Fasting insulin was associated with antenatal diet (p<0.05) but not postweaning diet (p=0.0777) and there was no interaction between them. Antenatal dietary-restriction and postweaning ω-3 diet did not significantly change fasting serum insulin levels in offspring. Insulin incremental peak at 30 min was associated with maternal diet (p<0.05) but not postweaning diet and there was no interaction between the two diets. Dietary-restriction elevated the peak insulin response compared to offspring from control dams though both were fed the postweaning control diet (R/C: 0.12 ± 0.048 ng/mL; C/C: 0.024 ± 0.017 ng/mL; p<0.05). The postweaning ω-3 diet decreased insulin peak in dietary-restricted mice (R/ω3: 0.03 ± 0.108 ng/mL, p<0.05), but had no effect on antenatal control offspring.

Figure 4.7D shows incremental serum insulin AUC at 12 months of age. Insulin AUC did not vary with antenatal and postweaning diets, and there was no interaction between the diets. Antenatal dietary-restriction increased insulin AUC, but not significantly. As well, a postweaning ω-3 diet did not significantly impact insulin AUC.

HOMA-IR, at 3 and 12 months of age, was significantly associated with postweaning diet (p<0.05, Figure 4.8A), but not with maternal diet (Figure 4.8B). Antenatal dietary restriction did not significantly affect HOMA-IR in offspring fed the control post-weaning diet (Figure 4.8A,B). However, the postweaning ω-3 diet reduced HOMA-IR in antenatal restriction offspring at both 3 and 12 months (R/ω3 vs R/C: p<0.05, Figure 4.8A,B). At 3 months, the
postweaning ω-3 diet also decreased HOMA-IR in offspring from control dams (C/ω3 vs C/C: p<0.05, Figure 4.8A) and this trend was maintained at 12 months but was not significant (Figure 4.8B).

Figure 4.6: **Glucose tolerance testing at 3 months.** Serum glucose (A), glucose AUC (B), serum insulin (C) and insulin AUC (D) during glucose tolerance testing at 3 months of age in offspring of mothers fed control (C) or restricted (R) diet during pregnancy and fed a control or omega-3 (ω-3) diet from weaning. Data are means ± SEM, n values on histograms represent the numbers of males and of whole litters. Histograms with different letters are significantly different (p<0.05).
Figure 4.7: **Glucose tolerance testing at 12 months.** Serum glucose (A), glucose AUC (B), serum insulin (C) and insulin AUC (D) during glucose tolerance testing at 12 months of age in offspring of mothers fed control (C) or restricted (R) diet during pregnancy and fed a control or omega-3 (ω-3) diet from weaning. Data are means ± SEM, n values on histograms represent the numbers of males and of whole litters. Histograms with different letters are significantly different (p<0.05).
HOMA-IR at 3 (A) and 12 months (B) in offspring of mothers fed control (C) or restricted (R) diet during pregnancy and fed a control or omega-3 (ω-3) diet from weaning. Data are means ± SEM, n values on histograms represent the numbers of males and of whole litters. Histograms with different letters are significantly different (p<0.05).

4.4. Discussion

The present study was designed to measure the effects of a diet rich in ω-3 FA fed to male offspring programmed (as a result of maternal undernutrition) for fetal growth restriction and symptoms of the metabolic syndrome in adulthood [85, 106]. In the preliminary study, the ω-3 diet did not affect caloric intake in B6 males at 8 weeks of age indicating that there is no difference in diet consumption between antenatal dietary restricted and antenatal control offspring. Increased dietary ω-3 FA in early postnatal life prevented the development of obesity, reduced glucose intolerance and improved insulin sensitivity in male offspring from dietary restricted dams. The ω-3 diet also increased insulin sensitivity in offspring from control dams, suggesting that ω-3 FAs are beneficial regardless of maternal dietary manipulation.

Dietary restriction elevated obesity in offspring that we have previously shown to be growth restricted in early life [85]. Cohort studies have correlated low fetal or newborn weights with obesity [275]. Animal studies have also demonstrated that offspring subjected to 50% and 70% maternal caloric restriction are more inclined to higher fat deposition in the presence of...
hypercaloric or high fat diets [93, 110]. There are several likely mechanisms leading to increased obesity in growth restricted offspring and we will discuss two of them. First, maternal undernutrition leads to decreased levels of fetal leptin (see Chapter 3), which is an adipocyte-derived hormone that regulates the hypothalamic satiety signal [184], impairs hypothalamic development and is associated with hyperleptinemia in later life [187, 189]. Although we did not measure dietary intake, Vickers et al. correlated a maternal 70% caloric restriction throughout gestation with obesity, hyperleptinemia, and with dysregulation of appetite in adult rat offspring in the form of increased caloric intake [110]. Hence, the dietary restricted offspring in our study may be obese because of the increased caloric consumption due to an inability to regulate appetite. Second, dietary restriction may increase obesity by elevating lipid synthesis and deposition. The dietary restriction increases the peak response to glucose after fasting at 3 months of age, which may indicate that these offspring have higher postprandial glucose levels compared to males from control dams. As a result, excess glucose may be converted into substrates for lipid synthesis. This process may be dependent on insulin-stimulated transcription of lipogenic genes, or insulin-independent and mediated by the carbohydrate response element binding protein that that directly responds to glucose levels to coordinate lipogenesis [276]. Obesity is a multifactorial syndrome that can arise from environmental, behavioural and genetic factors [277]. Therefore, the dietary restriction in our study probably increases obesity through a combination of mechanisms.

The observation that restricted offspring fed the postweaning ω-3 diet were less obese compared to postweaning control offspring may be because they were prevented from developing hyperleptinemia [267]. We found growth-restricted fetuses from malnourished dams had reduced leptin levels in late gestation (see Chapter 3). Since leptin is directly linked to appetite regulation, the absence of hyperleptinemia would suggest the improved ability to regulate food intake. Wyrwoll et al. found that fetal glucocorticoid exposure during late gestation programmed hyperleptinemia and hypertension, both of which were prevented by increasing postnatal ω-3 FA dietary intake [267]. Therefore, perhaps the ω-3 diet in our study reduces obesity by improving leptin sensitivity and the ability to control diet intake in dietary restricted males.
At 3 months of age, maternal undernutrition altered glucose and insulin response to glucose load following overnight fasting in offspring compared to males from malnourished dams only, which correlates with our previous findings [106]. Dietary restricted males do not handle the glucose challenge as well as antenatal controls, and this correlates with findings that maternal undernutrition leads to glucose intolerance and reduced insulin sensitivity in human and animal studies [62, 94, 201]. Dietary restriction increases obesity, which contributes to the pathogenesis of insulin resistance by activating inflammatory processes in several ways that inhibit insulin signalling [14, 232]. In obesity, increased adipocyte numbers, hepatic uptake of free fatty acids, and recruitment of macrophages to the adipose tissue and liver results in overproduction of secreted pro-inflammatory cytokines, such as leptin, TNF-α and IL-6 [21, 232]. These proinflammatory cytokines act on neighbouring cells to initiate intracellular inflammatory pathways that lead to systemic insulin resistance [21]. Hence, the elevated obesity seen in dietary restricted males may have contributed to their inability to handle the glucose challenge unlike offspring from control dams.

Postweaning control offspring from dietary restricted or control dams had similar fasting insulin, fasting glucose and HOMA-IR levels, possibly because the 30% caloric restriction is not a sufficient challenge to fully induce the metabolic syndrome phenotype. Rodent studies often introduce a global caloric restriction during gestation followed by a secondary challenge, such a high-fat postnatal diet, which results in male offspring developing metabolic disease symptoms to a greater degree than those offspring exposed only to the prenatal caloric restriction [110]. Supporting this theory, HOMA-IR levels were not affected by the dietary restriction despite increased glucose AUC and insulin AUC. This implies that restricted males are glucose intolerant with higher insulin response to the glucose challenge but do not develop insulin resistance to the fullest degree. Based on our findings, future studies should determine whether dietary restricted offspring are more vulnerable to a secondary challenge, such as a hypercaloric or high sucrose diet, and develop a more severe metabolic syndrome phenotype compared to antenatal control offspring groups.
A postweaning diet rich in ω-3 FA has positive effects on glucose and insulin response following an intraperitoneal glucose load in dietary-restricted B6 mice. The associated decrease in HOMA-IR and insulin AUC indicates that they required less insulin to manage the glucose challenge, suggesting that increased consumption of dietary ω-3 FA improves insulin sensitivity. Increased dietary ω-3 FA have a wide range of benefits, from preventing rats from developing insulin resistance and other symptoms of metabolic disease that are induced by high-fat or high-sucrose diets [278, 279], to reducing plasma triglycerides in overweight patients [161, 163]. Although we did not measure triglyceride concentrations in tissue or serum, studies show that maternal undernutrition increases circulating free fatty acid and triglyceride levels in fetuses and adult rodent offspring, thereby increasing their availability for uptake in insulin-sensitive tissues, such the liver and skeletal muscle [263, 280]. This reduces insulin sensitivity and insulin resistance subsequently develops [263, 280]. Rodent studies have found the fish oil diet, high in ω-3 FA, to improve several indices of metabolic disease in insulin-sensitive tissues compared to rodents fed high-fat or control diets. The fish oil diet lowered hepatic and intramuscular triglyceride content, and reduced plasma triglyceride levels [280-282], decreases hepatic lipid content by increasing hepatic excretion of macrophage-derived and HDL-derived cholesterol [283], lowers expression of enzymes involved in hepatic lipogenesis [279, 283, 284] and intramuscular insulin-stimulated glycogen synthesis and glucose disposal [282]. As discussed above, insulin resistance is a product of inflammatory pathway activation [21, 232]. An in vivo study by Oh et al., found that the G-protein coupled receptor, GPR120, is found in proinflammatory macrophages and adipocytes and senses ω-3 FA [31]. The ω-3 FAs stimulate signalling through GPR120 to mediate anti-inflammatory and insulin-sensitizing effects [31]. These animal and in vivo studies suggest that the ω-3 diet improves glucose and insulin response to glucose load in our dietary restricted males by reducing triglyceride accumulation in insulin sensitive tissues and by blocking the inflammatory processes that inhibit insulin signalling.

Dietary restricted males had similar HOMA-IR, glucose AUC and insulin AUC between control and restricted offspring by 12 months of age. We propose two possible explanations for the similarities in glucose AUC and insulin AUC at 12 months between offspring from antenatal control and dietary-restricted dams that were fed the postweaning control diet. First, by 12
months of age the number of animals within the litters were reduced to as low as one male per litter, which may have decreased the power of the statistical test leading to insignificant differences. Second, the B6 mouse strain are inherently inclined towards developing metabolic disease, as they are highly susceptible to diet-induced diabetes, hyperleptinemia and obesity, and are less glucose tolerant and insulin sensitive compared to other strains, such as C3H/He, 129/Sv and A/J [285-289]. Indeed, if B6 spontaneously track towards the metabolic syndrome in the absence of antenatal dietary manipulation, then the ω-3 FA might improve insulin sensitivity in offspring from control dams in the same manner as dietary restricted offspring fed the postweaning ω-3 diet. On the other hand, if the controls already have normal levels of insulin and glucose sensitivity, then our findings suggest caution when considering the dosage and timing of introducing ω-3 FA as part of a nutritional intervention in early development. Docosahexanoic acid, an ω-3 FA, is essential for proper fetal retinal and cognitive development [290, 291]. Realizing this, the World Association of Perinatal Medicine Dietary Guidelines Working Group currently recommends the inclusion of DHA in infant formulas [292]. Several animal studies have introduced ω-3 FA into maternal diet during different perinatal period with varying results, largely due to inconsistencies in methodology between authors [293]. We have a well-established mouse model for maternal undernutrition and future studies may be interested in investigating how introducing the ω-3 diet at different perinatal periods affects development of the metabolic syndrome in male offspring. Unfortunately, at this time it is difficult to clearly state why offspring from control dams are similar to dietary restricted males for some indices of glucose and insulin sensitivity.

A postweaning diet enriched with ω-3 FA improved glucose tolerance, insulin resistance and obesity in antenatal dietary restricted male offspring. In addition, it improved glucose tolerance, and insulin sensitivity in offspring from control dams. To our knowledge there are no other maternal undernutrition studies that have investigated early postnatal life intervention with dietary ω-3 FA to prevent the development of the metabolic syndrome.
Chapter 5

Summary and Significance

5.1 Summary of Results

We successfully re-established the 30% maternal undernutrition protocol in a new animal facility. Dams subjected to dietary restriction during pregnancy weighed less than dams allowed *ad libitum* access to food in late gestation. They produced fetuses with reduced body weights and increased placental weight:bodyweight ratios consistent with our previous findings [85].

Maternal undernutrition also altered late gestation fetal glycogen and glucose metabolism in the liver. The rate of fetal hepatic glycogen synthesis was decreased in response to maternal undernutrition in comparison to fetuses from control-fed mothers. Dietary restricted male fetuses had slight, but significant, increase in expression of *Phka2, IRS1*, and *IRS2* when compared to control male fetuses. These are genes that encode enzymes involved in insulin signalling, and glycogen/glucose metabolism. In this cohort of male fetuses used to measure hepatic gene expression, dietary restriction did not alter glucose and insulin levels. Hepatic gene expression was not measured in female fetuses. After treating fetal liver tissue (pooled for each litter) with insulin, there were no differences observed in protein levels of phospho-IR nor phospho-GSK3:total GSK3 ratio between dietary restricted and control fetal litters.

Dietary restriction may alter glucose, insulin and leptin levels in maternal and fetal circulation. Maternal and fetal glucose and insulin levels were impacted by maternal undernutrition in the mouse cohort used for the *in vitro* hepatic insulin challenge. Dams exposed to maternal undernutrition had lower maternal glucose, insulin and leptin levels when compared to control dams. They also had similar glucose:insulin ratios to control dams. In both male and female fetuses, dietary restriction lowered serum glucose, insulin and leptin levels but did not alter glucose:insulin ratio in comparison to control male fetuses. In the cohort of mice used to measure fetal hepatic gene expression, the caloric restriction lowered maternal glucose, insulin,
glucose:insulin ratio and leptin in comparison to control-fed dams. However, dietary restriction did not alter fetal glucose and insulin levels in this group of mice.

This thesis focused on male offspring because we have previously found females to be less susceptible to developing symptoms of the metabolic syndrome in response to maternal dietary restriction. In adulthood, male offspring of dietary restricted mothers developed obesity, insulin resistance and glucose intolerance [106]. At 3 months of age, both dietary-restricted and control offspring had similar body weights. At 12 months of age, dietary restricted offspring were heavier with a higher percentage of body fat and increased fat:lean ratio compared to control males. HOMA-IR, fasting glucose levels, and fasting insulin levels were similar between the dietary groups at both 3 and 12 months of age. At 3 months of age, male offspring of dietary restricted dams showed indications of glucose intolerance and insulin resistance following GTT. Dietary restricted male offspring had higher glucose and insulin AUC, and higher peak glucose and insulin response during GTT compared to males from control-fed mothers. At 12 months of age, glucose and insulin AUC were not different between dietary restricted and control offspring, although there was a higher peak insulin response to GTT in dietary restricted males compared to control males. Therefore, maternal undernutrition resulted in male offspring developing indications of glucose intolerance and insulin resistance compared to control males at 3 months of age, which were not observed at 12 months, although dietary restricted offspring had a higher percentage of body fat than control male offspring.

To determine whether an omega-3 (ω-3) diet in the postweaning period may prevent the adverse metabolic effects of dietary restriction in utero, we introduced a diet enriched in ω-3 fatty acids to male offspring from dietary restricted and control dams after weaning. A postweaning ω-3 diet protected dietary restricted male offspring from developing symptoms of the metabolic syndrome at 3 and 12 months of age. At 3 months of age, R/ω3 males had lower body weights when fed the postweaning ω-3 diet than R/C male offspring, but this weight difference was not observed at 12 months. At 12 months, R/ω3 males had lower percentages of body fat and a non-significant trend towards decreased fat:lean ratio in comparison to C/C males. At 3 months of age, R/ω3 offspring had lower fasting glucose levels but similar fasting insulin levels compared
to R/C offspring. At both 3 and 12 months of age, HOMA-IR was reduced in R/ω3 males compared to R/C males. Fasting glucose and insulin levels did not differ between R/C and R/ω3 offspring at 12 months of age. At 3 months of age, R/ω3 males had similar glucose AUC and glucose peak response to GTT compared to R/C males. R/ω3 males also had, lower insulin AUC, and a decreased insulin peak response to GTT in comparison to R/C offspring at 3 months. At 12 months of age during GTT, glucose AUC and insulin AUC did not differ between dietary restricted offspring, regardless of postweaning diet. The ω-3 diet did, however, decrease insulin peak response to GTT, but had no impact on glucose peak response compared with controls at 12 months. At 3 months of age, R/ω3 males had improved indicators of insulin resistance compared to R/C, a few of which were also observed at 12 months.

Interestingly, control offspring also benefitted from being fed the ω-3 diet postweaning. At 3 months of age, C/ω3 offspring had lower body weights than C/C offspring, but this difference disappeared at 12 months. C/ω3 and C/C offspring had similar percentages of body fat and fat:lean ratios at 12 months. At 3 months of age, C/ω3 males had lower HOMA-IR than C/C males, but they had similar HOMA-IR at 12 months. At 3 months of age, C/ω3 offspring had lower fasting glucose levels than C/C offspring, but this difference was not maintained to 12 months. Fasting insulin in male control mice did not vary with postweaning diet at either time point. At 3 and 12 months, C/ω3 offspring fed the ω-3 diet had similar insulin AUC and glucose AUC during GTT as C/C offspring. Peak glucose and insulin levels in response to the glucose challenge in GTT were not altered in control offspring by postweaning diet. Therefore, the ω-3 diet reduced body weight, lowered glycemia, and improved the HOMA-IR index of insulin sensitivity in males from control-fed dams at 3 months, however these changes were not observed at 12 months of age.

5.2 Significance

Altered glycogen synthesis and changes in hepatic expression of genes involved in glucose metabolism suggest that maternal undernutrition impairs glycogen metabolism and upregulates glucose production in male fetuses. If gene expression directly correlated with enzymatic activity, there may be increased fetal hepatic glycogen breakdown and increased hepatic insulin
sensitivity in dietary restricted males compared to male fetuses from dams fed ad libitum. Maternal undernutrition rat models have shown increased hepatic gluconeogenic PEPCK enzyme activity and gene expression in males compared to control male offspring [132, 133]. If these observations could be related to humans, the fetus may adapt to nutrient deprivation from mothers by increasing hepatic glucose production. This alteration may then be maintained postnatally leading to elevated glucose synthesis that may contribute to the development of glucose intolerance and insulin resistance with age. Perhaps then the fetus adapts to low nutrient conditions by increasing insulin sensitivity because it expects the extra-uterine environment to be one of reduced food. The fetus may be readying itself for the occasional times when food is plentiful so that it can easily stimulate the insulin signalling pathway to increase its glycogen stores. Our data indicates that maternal undernutrition may affect the hepatic insulin signalling pathway and this change in expression may somehow contribute to the offspring predisposition towards the metabolic syndrome. This is interesting because insulin resistance is considered to be largely obesity-induced. Here, our observations suggest alterations in insulin sensitivity prior to the development of obesity. One weakness of this study is that we did not investigate female fetuses further in terms of hepatic enzyme gene expression, especially since this cohort of dietary restricted females had higher glucose:insulin ratio as mentioned above. Future experiments may further investigate the female fetal response to maternal undernutrition. The in vitro hepatic insulin challenge requires further optimization in order to evaluate whether the insulin signalling pathway activation is altered in dietary restricted fetuses compared to control fetuses.

Reduced fetal body weights and increased placental weights in response to maternal undernutrition demonstrate the impact of maternal nutrition on fetal growth. Maternal nutrition is a large determinant of fetal growth [90, 168] that contributes to the offspring’s risk of developing metabolic disease. In our mouse model, dietary-restricted males with low body weights later develop symptoms of the metabolic syndrome [85, 106]. Consistent with our findings in rodents, maternal undernutrition during specific periods of gestation can lower birth weight in humans [62, 63]. Low newborn birth weights are associated with a higher incidence of metabolic disease in adulthood [61, 62]. Hence, a moderate caloric restriction from mid-late gestation in mice may be a useful animal model for studying the developmental origins of disease in humans. If so, our
findings emphasize the importance of adequate maternal dietary intake during pregnancy. On the other hand, maternal overnutrition would not be recommended since maternal obesity is linked to gestational diabetes and fetal overgrowth in humans [294]. If our observations in mice could be applied to humans, we would emphasize the importance of monitoring both maternal nutrition and growth during pregnancy. Also, low fetal body weights and increased placental weights may be useful as markers in predicting the offspring’s risk of developing metabolic disease in later life. Both measurements may be indicators of abnormal fetal growth and increased risk of developing the metabolic syndrome. A limitation of this study was that we did not conduct an in-depth analysis of the placenta. The placenta plays a large role in determining nutrient supply to the fetus [295]. Placental morphology and expression of nutrient transporters are altered in some animal models of maternal undernutrition [117]. In the future, we might measure these indices to determine whether dietary restricted fetal placentae adapt to maternal undernutrition in a manner that further affects fetal nutrition in our mouse model.

Maternal dietary restriction reduced fetal glucose and insulin levels compared to controls (Chapter 3), which may alter fetal perception of the external environment. In humans, the concentration of glucose that crosses the placenta into the fetal circulation at term varies with maternal glucose concentration [204]. If mice are similar to humans, then low fetal glucose levels may be a result of reduced transfer of maternal glucose into fetal circulation of dietary-restricted mice. In mice and humans, glucose levels in the fetus regulate insulin secretion [86]. Thus, low glucose levels may account for the low insulin levels in dietary-restricted fetuses compared to controls. Based on low glucose provision from dams, the dietary-restricted fetal mouse may predict the external environment to be low in glucose, or low in nutrition, and adapt its metabolic development accordingly. This may explain the reduced capability of R/C males to handle the glucose load in GTT compared to C/C males in adulthood. If these findings hold true for humans, our observations support the established hypothesis that the intrauterine environment shapes fetal development [69].

In Chapter 2, in spite of reduced maternal glucose with dietary restriction, dietary restricted fetuses did not have different glucose and insulin concentrations compared to control fetuses,
which is dissimilar to our findings in Chapter 3. Future studies may investigate other factors that potentially account for the difference between dams and their fetuses in terms of their glycemic states. Together, Chapters 2 and 3 show that maternal nutrition may contribute to fetal nutrition but that the two do not necessarily need to show the same trends. The discrepancies between cohorts in Chapters 2 and 3 may be a consequence of different animals being bred at different times. The differences in glucose and insulin concentrations between the two cohorts of mice might be explained in part by different methods of analysis. Glucose concentrations were measured in fetal serum in Chapter 2 and in fetal whole blood in Chapter 3, which is additional limitation of this thesis. The dietary restriction cohort should be replicated a third time with adequate animal numbers to further determine the trend in fetal glucose and insulin levels.

Fetal leptin levels are reduced by maternal undernutrition and are associated with small fetal body weights and the development of obesity in later life. Leptin modulates fetal growth through its receptors, which are expressed in various fetal tissues in the mouse [216, 217, 296]. Similarly in humans, low fetal leptin levels may contribute to the reduced fetal growth that is observed in IUGR newborns [186, 213]. We observed that reduced fetal leptin levels are associated with obesity in adulthood, a correlation also found in humans [186, 187]. Thus, leptin’s role in growth and development is similar in humans and mice. Rodent models have shown that leptin in the fetal circulation stimulates the development of the hypothalamus, which is involved in postnatal appetite regulation [189]. Decreased fetal leptin levels in the circulation may alter the ability to regulate food intake and energy expenditure demands in later life [187]. Vickers et al. (2000) demonstrated that rat offspring exposed to maternal undernutrition had lower birth weights and higher food intake throughout life [110]. If these findings hold true for human populations, low leptin levels in newborns may serve as a marker of a decreased ability to regulate appetite in later life. It might be recommended that the dietary intake of these infants be monitored closely throughout their lives since they may have a higher inclination towards excessive food intake and obesity.

In B6 mice, our observations that male and female fetuses responded differently to maternal undernutrition are consistent with human populations. Late gestation control males were heavier
than females in Chapter 3, which is a relationship observed in human newborns [297]. While both sexes were growth-restricted as a result of maternal dietary restriction, males were more susceptible to developing metabolic disease than females [106]. Similarly in human populations, reduced body size at birth is associated with a higher risk of developing coronary heart disease, lower insulin sensitivity and hyperinsulinemia in men than in women [298, 299]. There are other sex-specific adaptations to maternal stressors as observed in the development of hypertension in rat offspring of dams fed a low protein diet [140]. In the Dutch famine human cohort, maternal undernutrition was associated with sex-specific differences in the atherogenic lipid profiles of middle-aged offspring [300]. The mechanisms underlying sex differences in pregnancy are unclear at this time. The differential effects that maternal dietary restriction has on male and female developmental physiology are areas for future study.

A postnatal diet enriched in ω-3 FAs may prevent the development of metabolic disorders associated with maternal undernutrition in mice. Other animal models have investigated the use of ω-3 diets to treat insulin resistance and obesity with success. For example, a study by Wywroll et al. (2006) showed that a postweaning diet enriched with ω-3 FAs prevented the programmed development of hyperleptinemia and hypertension in adulthood [267]. We showed that ω-3 fatty acids prevented development of signs associated with the metabolic syndrome following maternal undernutrition. Studies have shown the benefits recommending increased ω-3 FA consumption to patients in a clinical setting. For example, overweight patients on an energy-restricted diet showed higher weight loss after including a fish meal as part of their daily diet [161]. If our results hold true for human populations, we might advocate the implementation of ω-3 FA supplementation during early postnatal development for offspring exposed to maternal undernutrition. However, much more work needs to be done to fully understand the mechanisms underlying the physiological effects of ω-3 FAs. We found that an ω-3 diet lowered body weight at 3 months of age in offspring from control-fed dams. This reduced body weight with increased ω-3 FA intake in controls disappeared by 12 months of age. It is unclear how ω-3 FA supplementation in controls impacts their development. Thus, our results indicate that caution should be taken when there is consideration of providing ω-3 FA supplements to children as they are still developing. Although we measured dietary intake in a small group of adult males to
ensure that the \(\omega-3\) diet was palatable, we did not measure dietary intake throughout our study. It would have been of interest to determine whether maternal undernutrition lead to dysregulation of appetite in adult offspring. Another limitation of this study is the low animal numbers at 12 months of age, which reduced the power of our statistical analysis. Furthermore, the \(\omega-3\) diet contained approximately 35\% of fat content as \(\omega-3\) FAs that may be difficult to attain in the Western diet. Future studies may be interested in mixing the control and \(\omega-3\) diet to lower the percentage of \(\omega-3\) FA consumption and determine the lowest level necessary for benefit in dietary restricted males.

We acknowledge that the low animal numbers in our experiments limits our studies and findings. Retrospective power calculations were performed which indicated that an increase in animal numbers might have produced statistically significant differences between the dietary groups of mice. Further, we found different fetal glucose and insulin levels in two of our maternal undernutrition cohorts. We are unable to explain at this time the discrepancy in these results or their impact on the developing fetus. Finally, we measured gene expression, but not activity of enzymes involved in glucose metabolism, which may not be representative of functional changes in enzymes and proteins. A complete analysis of protein expression and enzyme activity should be considered for future studies.

In the absence of human models, mice serve as an appropriate model for studying disease in a more invasive and ethical manner [99, 100]. We have previously reported that a moderate maternal caloric restriction from mid- to late gestation produces growth restricted C57BL/6J fetuses [85]. Dietary restricted male offspring later develop indications of insulin resistance, glucose intolerance and obesity [106]. In this thesis, we investigated the potential mechanisms underlying the onset of these metabolic diseases in fetal life and used a postweaning nutritional intervention to prevent their postnatal development. These findings may have implications for maternal nutrition and health and disease outcome in offspring.
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